

ISOLATION, CHEMICAL MODIFICATION AND APPLICATIONS OF FLAX CYCLOLINOPEPTIDES

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By

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ABSTRACT

Oil from flaxseed (*Linum usitatissimum* L.) contains hydrophobic cyclic peptides or cyclolinopeptides (CLs) comprising eight or nine amino acids. These bioactive compounds have potential therapeutic applications and may be used as scaffolds for increased utility. Two steps were undertaken to increase the potential utility of these compounds. Initially multigram quantities of flax CLs were highly enriched from flax oil. Subsequently new synthetic procedures were developed for modification of the CLs through the methionine group (Met). Finally, the utility of the modified CLs was tested in a number of applications. CLs were recovered from a crude oil extract that contain five CLs (CLA, CLC, CLE, CLJ and CLK). Oxidation of this mixture reduced the complexity of the mix to just three CLA, CLJ and CLK. CLJ and CLK were enriched then characterized by NMR and MS-MS methods. CLs containing methionine sulfoxide groups (Mso), CLC and CLE were isolated from crude mixture then selectively reduced to afford Met containing analogs: CLB and CLE'. The Met of modified CLs was used as a point for attachment of tags and couplers for various applications. Cyclic peptide modification through Met groups has not been reported previously. Synthetic methods were devised to introduce activating functional groups such as -CN, -COOH, -OH and -NH₂ to the sulfur atom of Met. The modified CL conjugates were characterized using spectrometric techniques including 1D and 2D NMR spectrometry, as well as mass spectrometry. After activation the CLs were covalently linked to molecules or materials of interest including fluorescence tags (coumarin), affinity chromatography media and bovine serum albumin (BSA) for production of polyclonal antibodies. Fluorescence studies were performed in methanol, ethanol, dimethylformamide and acetonitrile to study the solvent effect. CLs attached to solid affinity matrix showed specific binding to apolipoprotein A1 after incubation with chicken serum. These CLs also act as haptens and have been used to couple BSA to produce polyclonal antibodies. Met modification was a satisfactory approach to produce a range of useful peptide products where more conventional methods of molecule attachment are not available.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ALA	α -linolenic acid
Apo A1	Apolipoprotein A1
BSA	Bovine serum albumin
CD	Circular dichroism
CI-ELISA	Competitive indirect enzyme linked immunosorbent assay
CL	Cyclolinopeptide
COSY	Correlation spectroscopy
CsA	Cyclosporin A
DAD	Diode array detector
ESI-MS	Electrospray ionization-mass spectrometry
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
ESI-TOF-MS	Electrospray ionization-time of flight-mass
FTIR	Fourier transform infrared spectroscopy
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatogram
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
Met	Methionine
Mso	Methionine sulfoxide
Msn	Methionine sulfone
NCI-ELISA	Non-competitive indirect enzyme linked immunosorbent assay
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
pAb	Polyclonal antibody
PBS	Phosphate buffer saline
SDG	Secoisolariciresinol diglucoside
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

Flaxseed oil (*Linum usitatissimum* L.) contains hydrophobic cyclic peptides or cyclolinopeptides (CLs) comprising eight or nine amino acids. To date, eleven CLs (CLA–CLK), having molecular weights of approximately 1 kDa have been isolated from flax oil. The CLs (CLA, CLC, and CLE) have immunosuppressant activity and induce apoptosis in nematodes and a cancer cell line. CLs are thus bioactive compounds with potential for use as therapeutics. Methods to isolate CLs from flax oil in multigram quantities were investigated. The isolated CLs containing methionine sulfoxide (Mso) will be chemically modified and specific changes to the methionine (Met) or Mso group in a systematic fashion, without changing other amino acids. The Met group in modified CLs will be used as a point for attachment of tags and couplers for various applications. Modification of cyclic peptides through the Met has not been reported previously. The CLs will be systematically linked to molecules or materials of interest including fluorescence tags, affinity chromatography media and bovine serum albumin (BSA). Specifically, synthetic methods will be devised to introduce different activating functional groups such as -CN, -COOEt, -OH and -NH₂ to the sulfur moiety of Met. In addition, these modified CLs will be completely characterized using spectrometric techniques including 1D and 2D NMR. The coupled CL-fluorescent dye can be used to monitor CL in biological samples. In addition, the CL affinity matrix can be used to purify CL-binding proteins from complex mixtures. Finally, polyclonal antibodies generated from CL-protein conjugate to develop competitive indirect enzyme linked immunosorbent assay (CI-ELISA).

1.1 Hypothesis

In the process of oil extraction from flaxseed CLs are dissolved in flax oil that has similar properties to low polarity organic solvents like ethyl acetate. Silica gel is a polar adsorbent that readily adsorbs polar molecules like CLs from low polarity organic solvents. It is expected that CLs that are bound to silica gel can be extracted and enriched from the silica gel

using polar organic solvents such as methanol or blends of methanol with less polar solvents such as chloroform and dichloromethane.

Second hypothesis

Amino acids that contain heteroatoms such as a Met group are known to be reactive and, it may be possible to modify Met without affecting the amides present in CLs. Reactive Met occur in CLs such as CLB, but may also be accessed by reduction of CLs containing Mso (e.g. CLC).

Third hypothesis

CLB related reduced Met-containing CLs, may be modified by reacting with activated haloalkyl groups such as iodomethylene. CLs produced in this way may contain reactive -NH₂, -COOEt and -OH groups, which can be used to selectively couple intact CLs with other reactive functional groups present in fluorescent labels, affinity media, and carrier proteins. Coupled CLs may be used for fluorescence energy transfer from a chromophore to a metal bound to a CL or for purification of proteins from crude cell extracts. In addition, coupled CLs may also be used to produce antibodies by utilizing the CL as a hapten.

Fourth hypothesis

The Met modified CLs may be converted to more stable methionine sulfone (Msn) CLs. Msn CLs are shown to be stable than Met in terms of thermal and chemical stability. This will help to produce stable Msn CLs containing reactive side chains of -NH₂, -COOEt and -OH groups.

1.2 Objectives

The objectives of this research are to:

- Develop methods for isolation and purification of CLs from flax oil in multigram quantities. Specifically, Mso containing CLC and CLE will be isolated from silica that was previously equilibrated with flax oil.
- Develop a synthetic strategy to chemically modify the reactive side chains of these CLs. These new CLs will be completely characterized using 1D and 2D NMR techniques.

- Develop coupling methods to attach the above-modified CLs to dyes, solid matrix and protein. Dye linked CLs can be used as fluorescent tags. CLs linked to a solid matrix will be used to purify CL-binding proteins from complex mixtures and CLs bound to proteins will be used to induce the production of antibodies.
- Develop synthetic strategy to oxidise Met CLs to more stable Msn derivatives.

CHAPTER 2

LITERATURE REVIEW

2.1 Flaxseed

Flax (*Linum usitatissimum* L.), a member of the family Linaceae, is cultivated for production of oil seeds and fiber, and is one of the oldest crops (Jhala & Hall, 2010). The proximate composition of flax averaged 40% fat, 20% protein, 30% total dietary fibre, 6% moisture and 4% ash. These compositions vary with seed processing, genetics, growing environment and method of analysis (Daun, et al., 2003). Flax is rich in polyunsaturated fatty acids (73%) followed by monounsaturated (18%) and saturated (9%) fatty acids (Cunnane, et al., 1993). The major polyunsaturated fatty acid in Canadian flax is α -linolenic acid (ALA, omega-3, 57%) followed by linoleic acid (omega-6). Variance in the ratio of fatty acids depends on the temperature and variety of flax (Morris, 2007).

Seed proteins are classified based on their solubility in aqueous solvents. Globulins are insoluble in water but soluble in concentrated salt solutions, while albumins are soluble in water or dilute salt solutions. Globulins (11–12S) and albumin (2S) type constitute the majority of seed proteins (Care, 1954). Seed globulins are high molecular weight proteins (252–294 kDa) constituting 66% of total protein and these proteins are rich in aspartic acids, glutamic acids and arginine. However, seed albumins are low molecular weight proteins of about 16 kDa molecular weight and constitute 20% of the total proteins. The albumins are richer in glutamic acids, while arginine content is similar to globulins. However, albumins contain half the aspartic acid observed in globulins (Bhatty, 1995). The amino acid composition of flax is similar to soybean protein, which is considered to be one of the more nutritious plant proteins. Both flaxseed and soy contains higher amounts of glutamic acid (+glutamine), aspartic acid (+asparagine), leucine and arginine. This high content of amides serves as storage role in the seeds by providing nitrogen for seedling growth and germination. The amino acids that are essential for human nutrition are shown in Table 2.1 (Morris, 2007; Wanasundara & Shahidi, 2003). However, lysine is the limiting amino acid with 60% less concentration as found in soy protein (Bhatty, 1995).

Flaxseed also contains both soluble and insoluble dietary fiber and their proportions range between 20:80 and 40:60 depending on the extraction methods and chemical analysis (Mazza & Oomah, 1995). The soluble fiber consists of mucilage gums and insoluble fiber consists of lignin and cellulose (Mazza & Biliaderis, 1989; Vaisey-Genser & Morris, 2003). Mucilage gums constitutes about 8% of flaxseed dry weight and they are made up of both neutral and acidic polysaccharides. The neutral polysaccharide consists of D-xylose, D-galactose and L-arabinose, while acidic consists of L-galactose, L-fucose, L-rhamnose and D-galacturonic acids (Westcott & Muir, 2003). When these gums are dissolved in water it becomes viscous. Cellulose is main plant cell wall carbohydrate and lignin is a highly branched complex present within the plant cell wall. Lignin provides rigidity and strength to the cell wall (Morris, 2007).

Flaxseed also contains lignan substances, which are formed by coupling of cinnamyl alcohols (Westcott & Muir, 2003). Secoisolariciresinol diglucoside (SDG) is a principal lignan and has shown to have phytoestrogen effects. Flaxseed lignan is present at higher concentrations (75–200 times) than in other lignan containing foods (Johnsson, et al., 2000; Mazur, et al., 1996). SDG is a principal lignan in flaxseed, a phytochemical antioxidant that acts as a precursor of mammalian lignans and a phytoestrogen. The mammalian lignans are produced by conversion of plant lignan by gastrointestinal microbiota (Adolphe, et al., 2010; Prasad, 2004; Sagggar, et al., 2010). SDG can prevent the development of atherosclerosis and diabetes in model animals (Fukumitsu, et al., 2008; Prasad, 1999, 2009) and additional benefits include lowering of cholesterol levels (Fukumitsu, et al., 2010). In addition, the other group of lignans identified in flax include aryltetralin and aryl-naphthalene (Westcott & Muir, 2003).

Flaxseed also contains cyanogenic glycosides. These glycosides contain a cyanohydrin that is stabilized by the linkage of a carbohydrate to the free alcohol. The glycosides can be hydrolysed by enzymes or under acidic conditions releasing the cyanohydrin, which decomposes to hydrocyanic acid and the resulting aldehyde or ketone. These glucosides are classified into monoglucoside and diglucosides. Monoglucosides (linamarin and lotaustralin) are present in developing embryos, germinating seeds, leaves and flowers while diglucosides (linustatin and neolinustatin) occurs in mature seeds and developing embryos (Westcott & Muir, 2003). Flaxseed contains flavonoids (flavone C- and O-glycosides) and phenolic acids (ferulic acid, chlorogenic acid, gallic acid and 4-hydroxy benzoic acid). Flaxseed also contains phytic acid and linatine.

Table 2.1 Amino acid composition of flaxseed (Bhatta & Cherdkiatgumchai, 1990; Friedman & Levin, 1989; Oomah & Mazza, 1993).

Amino acid	Brown flax (Norlin) g/100 g protein	Yellow flax (Omega) g/100 g protein	Soy flour
Alanine	4.4	4.5	4.1
Arginine	9.2	9.4	7.3
Aspartic acid	9.3	9.7	11.7
Cystine	1.1	1.1	1.1
Glutamic acid	19.6	19.7	18.6
Glycine	5.8	5.8	4.0
Histidine ¹	2.2	2.3	2.5
Isoleucine ¹	4.0	4.0	4.7
Leucine ¹	5.8	5.9	7.7
Lysine ¹	4.0	3.9	5.8
Methionine ¹	1.5	1.4	1.2
Phenylalanine ¹	4.6	4.7	5.1
Proline	3.5	3.5	5.2
Serine	4.5	4.6	4.9
Threonine ¹	3.6	3.7	3.6
Tryptophan ¹	1.8	NR ²	NR ²
Tyrosine	2.3	2.3	3.4
Valine ¹	4.6	4.7	5.2

¹Essential amino acids for humans.

²NR: not reported.

Phytic acid also binds to starch and proteins. Linatine is a glutamyl derivative of D-proline and acts as a vitamin B6 antagonist. In addition, flax contains minor amounts of vitamins such as vitamin E [tocopherols (α -, β -, γ -, δ -)] and vitamin K. Phosphorus and potassium constitutes major mineral components and other minerals reported include manganese, zinc and iron (Daun, et al., 2003).

Polyunsaturated fatty acids, lignan, mucilage and CLs are major functional classes of compounds present in flax. The edible oil, linseed oil or flaxseed oil is obtained by extraction of flaxseed. Flaxseed oil is an important health food product because of its high level of polyunsaturated fatty acids such as linoleic acid (C18: 2) and ALA (C18: 3). These fatty acids cannot be synthesized by the human body and are known to have physiological functions. They act as intermediates in production of hormones like eicosanoids which help in immune function and regulate inflammation in animals (Oomah, 2001). Consumption of flaxseed oil increases the concentration of ALA in human blood plasma (Barceló-Coblijn, et al., 2008; Kaul, et al., 2008). ALA is a precursor of the polyunsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that reduce the risk of cardiovascular diseases (Gebauer, et al., 2006).

2.2 Plant Cyclic Peptides

Plant cyclopeptides are made up of peptide bonds consisting of 2–37 proteinaceous or non-protein amino acids and mostly they are present in higher plants in L-configuration. For example, arenariphilin A isolated from *Arenaria oreophila* consists of two amino acid residues, *cyclo*-(Thr-Gly) (Jia, et al., 2003). The role of cyclic peptides in plants is not known, but cyclic peptides have biological activity in mammals. For example, diandrine A has shown antiplatelet activity and Segetalins A, B, G and H have shown estrogen activity (Hsieh, et al., 2004; Morita, et al., 1995; Tan & Zhou, 2006; Yun, et al., 1997). During the past half century, about 455 cyclopeptides have been identified from higher plants and they arise from 26 plant families, 65 genera and 120 species. These cyclic peptides were classified based on their skeleton and distribution in plants. They are divided into two classes homo- and heterocyclopeptides, based on their peptidic bonds i.e. made up of proteinogenic amino acid or produced from non-proteinogenic amino acids. These two classes are further divided into five subclasses based on number of rings. Finally, these subclasses are divided into eight types based on their source and

characteristics of rings (Figures 2.1 and 2.2). According to Tan and Zhou, 455 cyclopeptides are divided into these eight types (I, II, III, IV, V, VI, VII, VIII containing 185, 2, 4, 13, 9, 168, 23 and 51 number of cyclic peptides, respectively). Types I and VI contains largest number of peptides among other types (Tan & Zhou, 2006).

In a recent review it was suggested that the name Orbitides replace the term Caryophyllaceae-type homomonocyclopeptides (Type VI). Orbitides are circular peptides composed of five to twelve proteinogenic amino acids linked in an N to C terminal fashion. Orbitides have been identified in nine individual plant families including the Rutaceae, Phytolaccaceae, Euphorbiaceae, Annonaceae, Caryophyllaceae, Lamiaceae, Linaceae, Schizandraceae, and Verbenaceae (Arnison, et al., 2013).

2.2.1 Methionine Containing Peptides

Most of the peptides are rich in hydrophobic amino acids such as Pro, Phe, Ile, Leu, Val, Tyr and Trp. A review of plant cyclic peptides showed that very few had Met in their backbone structure (Tan & Zhou, 2006). Met containing orbitides isolated from higher plants are listed in the Table 2.2. Many of the Met peptides are also observed as the corresponding partially oxidized Mso form. But it is not certain, if Mso containing peptides are present naturally in plants or if they occur as an artifact of peptide isolation. Some of the examples include flax CLs (CLD', CLE', CLF' and CLG') detected in flax oil (Stefanowicz, 2001). These are reduced CLs of CLD, CLE, CLF and CLG.

2.2.2 Conformational Study of Cyclic Peptides (Orbitides)

The biological activity of the cyclic peptides is closely related to the conformational state of the molecule. This conformational study helps to predict the active binding site of the molecule. In general, a peptide backbone tends to be flexible with many possible conformers. However cyclic peptides present in higher plants are often rich in proline (Pro) residues. Hence the restricted rotation of the backbone is due to intramolecular hydrogen bonding and rigid Pro residues that may lock the conformation of the molecule (Morita, et al., 1999). Conformation of peptides can be determined in both solution and solid state by using NMR and X-ray diffraction. NMR protocols such as ^1H , ^{13}C , COSY, HSQC, HMBC, NOESY, TOCSY provide the ability to determine many aspects of conformation. The one dimensional NMR such as ^1H and ^{13}C helps in the assignment of protons and carbon atoms in the framework of organic compounds.

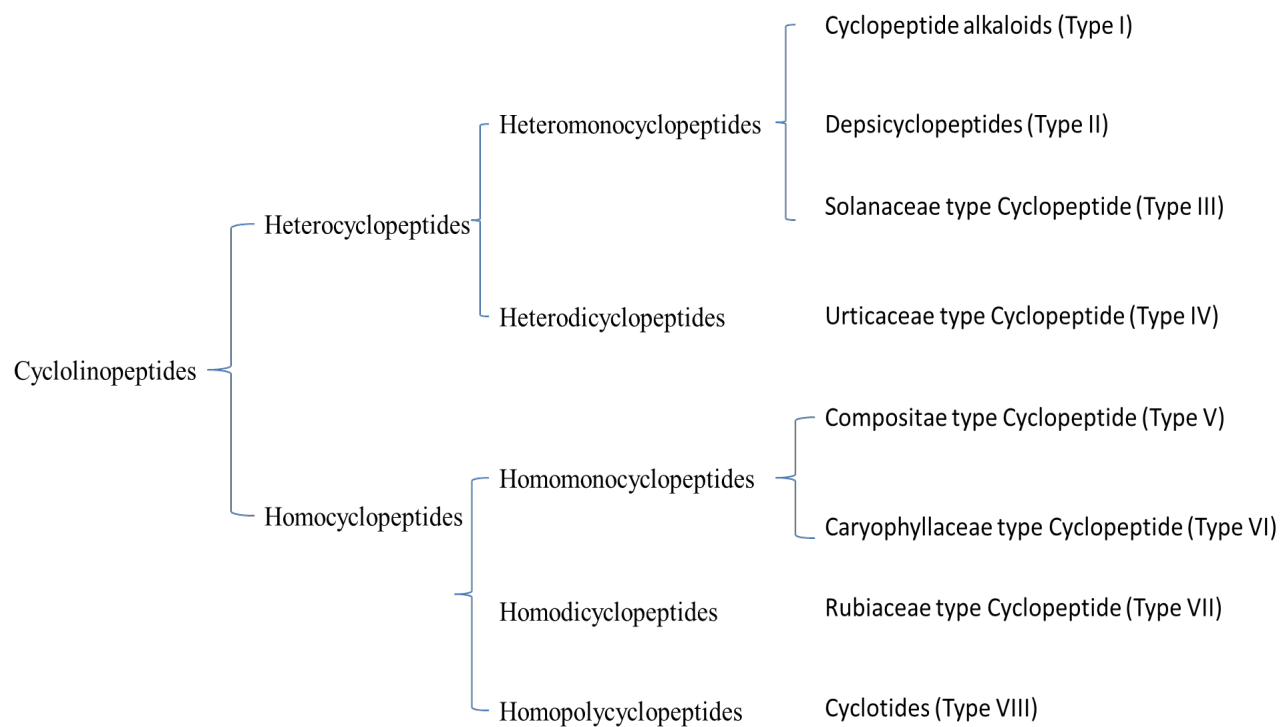
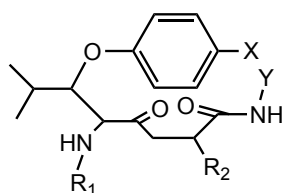
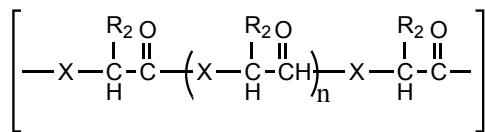


Figure 2.1 Classification of plant cyclic peptides. Adapted from (Tan & Zhou, 2006)



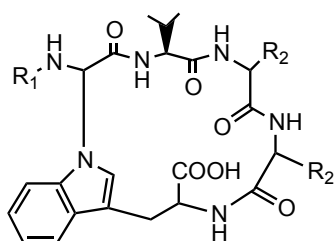
Type I



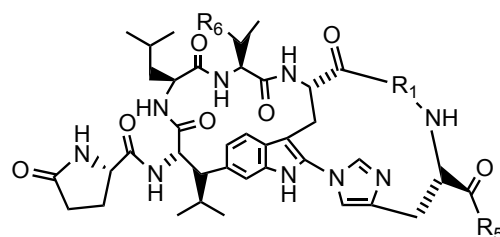
Type II: X=N, NH or O; n=4-5

Type VI: X=N, NH or O; n=0, 3-10

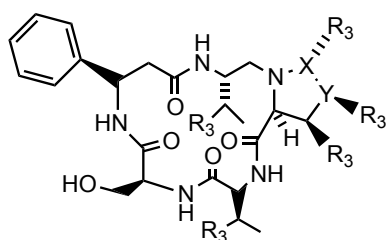
Type VIII: X=N, NH or O; n=12, 26-29, 32, 35



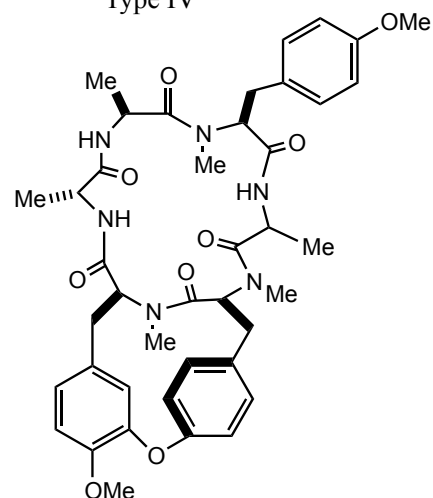
Type III



Type IV



Type V



Type VII

X-Y = CH=CH, CH(OH)-CH₂,
CH-CH, C=C, CH(OCH₃)-CH₂

R₁= amino acid residues

R₂= side chain of amino acids

R₃= H, OH, OCH₃, OAc, Cl, Oglc

R₄= NH₂, NHCH₃, N(CH₃)₂

R₅= OH or amino acid residues

R₆= CH₃ or CH₂CH₃

Figure 2.2 Structures of plant cyclic peptides. Adapted from Tan & Zhou (2006)

Table 2.2 Met containing cyclic peptides.

Cyclopeptide (Source)	Amino acid sequence (<i>cyclo</i> -)	Mol. formula
Cherimolacyclopeptide A (<i>Annona cherimola</i>)	Pro-Gln-Thr-Gly- Met -Leu-Pro-Ile	C ₃₈ H ₆₃ N ₉ O ₁₀ S
Cyclosquamosin B (<i>A. squamosa</i>)	Pro-Pro-Ile-Thr-Gly-Leu- Met -Gln	C ₃₈ H ₆₃ N ₉ O ₁₀ S
Cyclosquamosin G (<i>A. squamosa</i>)	Pro- Met -Thr-Ala-Ile-Val-Gly-Tyr	C ₃₉ H ₆₀ N ₈ O ₁₀ S
Chevalierin A (<i>Jatropha chevalieri</i>)	Gly-Ile-Pro-Ile-Leu-Ala-Ile- Met	C ₃₉ H ₆₈ N ₈ O ₈ S
Cycloleonuripeptide A (<i>L. heterophyllus</i>)	Gly-Pro-Pro-Pro-Tyr-Pro-Pro- Met -Ile	C ₄₇ H ₆₇ N ₉ O ₁₀ S
Cyclolinopeptide B (<i>Linum usitatissimum</i>)	Met -Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	C ₅₆ H ₈₃ N ₉ O ₉ S
Cyclolinopeptide D' (<i>Linum usitatissimum</i>)	Met -Leu-Leu-Pro-Phe-Phe-Trp-Ile	C ₅₇ H ₇₇ N ₉ O ₈ S
Cyclolinopeptide E' (<i>Linum usitatissimum</i>)	Met -Leu-Val-Phe-Pro-Leu-Phe-Ile	C ₅₁ H ₇₆ N ₈ O ₈ S
Cyclolinopeptide F' (<i>Linum usitatissimum</i>)	Met -Leu- Met -Pro-Phe-Phe-Trp-Val	C ₅₅ H ₇₃ N ₉ O ₈ S ₂
Cyclolinopeptide G' (<i>Linum usitatissimum</i>)	Met -Leu- Met -Pro-Phe-Phe-Trp-Ile	C ₅₆ H ₇₅ N ₉ O ₈ S ₂
Cyclolinopeptide H (<i>Linum usitatissimum</i>)	Mso-Leu- Met -Pro-Phe-Phe-Trp-Ile	C ₅₆ H ₇₅ N ₉ O ₉ S ₂
Cyclolinopeptide I (<i>Linum usitatissimum</i>)	Met -Leu-Mso-Pro-Phe-Phe-Trp-Val	C ₅₅ H ₇₃ N ₉ O ₉ S ₂

However, chemical shifts similarity of different amino acid residues may result in spectral overlap making ^1H and ^{13}C assignments difficult (Silverstein, 2005). Multi-dimensional NMR protocols such as COSY, HSQC, HMBC, NOESY, ROESY and TOCSY may be employed to determine and identify the relationships of protons in different environments. The tertiary structure of the polypeptide chains is determined using Nuclear Overhauser Enhancement (NOE) spectroscopy. NOE gives information about the protons, which are distant from each other but in close ($< 5\text{\AA}$) space (Pomilio, et al., 2006). For example, the protons attached to the heteroatoms were assigned from ^1H NMR, and their coupling to amide protons and carbonyl carbons employed NOE and HMBC correlations. These spectra provided the amino acid sequence of the cyclic peptide. Sequential assignment of β , γ and δ protons were done using ^1H - ^1H COSY and elucidation of bonds between these protons and carbon atoms was performed by HSQC. X-ray crystallography provides the static structure of the molecule in a crystal and it is complementary to NMR studies but the information gathered from X-ray and NMR may differ. The structure analysis of compounds by X-ray diffraction requires either a high quality crystal or a powder. The densities of electrons surrounding atoms in the crystal diffract the path of the X-rays in a pattern that can be interpreted to provide the structure. This technique can be applied to macromolecules ($> 100\text{ kDa}$). In contrast, the conformational environment of individual atoms in dissolved molecules may be monitored with NMR. The development of cryogenic NMR has made them more sensitive towards higher molecular weight biomolecules. However both techniques are complementary to each other and have been used together to determine molecular structure (Brünger, 1997; Feng, et al., 2011; Sikic, et al., 2010). Some of the examples of cyclic peptides involving structure determination in both solid and solution states are: - cyclolinopeptide A has shown five intramolecular hydrogen bonds and four turns with a cis geometry between Pro-Pro bond in the crystal structure, while the other peptide bond has a trans geometry. The solid-state structure of CLA was obtained in different mixture of solvents such as isopropanol-water, methanol-water and methanol-isopropanol. The crystal structures show inclusion of solvent molecules through hydrogen bonding (Di Blasio, et al., 1989; Matsumoto, et al., 2002; Quail, et al., 2009). However, solution state of CLA was determined in DMSO, methanol, isopropanol and chloroform. Various temperatures were used to minimize the conformers. NMR studies at 214 K in chloroform shows similar conformational structure as solid state. (Di Blasio, et al., 1989; Matsumoto, et al., 2002). Yunnanin A has also

shown similar conformation in both solid and solution state. It shows three intramolecular hydrogen bonds with two β turns and β -bulge unit with trans amide bonds (Morita, et al., 1997). However, Segetalin A shows different conformation in solid and solution state. In solid state, molecule shows two β turns (types I and VI) fixed by two trans-annular hydrogen bonds between Val and Gly. But there were two types (II and VI) of β turns present in solution state. This result shows that Segetalin A took different backbone conformations in solution and solid state. (Morita, et al., 1995).

2.2.3 Biological Activity

The cyclic peptides belong to orbitide family have shown interesting biological activities such as antimalarial, immunomodulating, immunosuppressive, antiplatelet and antifungal (Arnison, et al., 2013). Some examples of plant hydrophobic cyclic peptides are: - Cycloleonurinin is a cyclic dodecapeptide isolated from fruits of *Leonurus heterophyllus* that has shown potent suppression of human peripheral blood lymphocyte proliferation and, therefore, may be immunosuppressive. It showed the inhibitory effects on concanavalin A stimulated human peripheral blood lymphocytes with an IC_{50} of 28 ng/mL, which is comparable to well known immunosuppressive agent, cyclosporine A (IC_{50} 3 ng/mL) (Morita, et al., 1997). Curcacycline B is a cyclic nonapeptide isolated from *Jatropha curcas* latex has shown to increase the rotamase activity of human cyclophilin B i.e interconversion of cis and trans isomers of peptidyl-prolyl amide bonds. This peptide contains one proline and act as peptidyl-prolyl cis-trans isomerase (PPIase) substrate by increasing PPIase activity of cyclophilin-B of 60% at 30 μ m (Auvin, et al., 1997). Diandrine A was isolated from the plant *D. diandra* has shown antiplatelet activity. It inhibits platelet aggregation induced by collagen and the motif of Gly-Pro-Trp-Pro may interfere with the interaction between collagen receptor and collagen, but the mechanism has not been discussed (Hsieh, et al., 2004). Since collagen plays an important role in platelet aggregation by providing the site for platelet adhesion and also stimulating the secretion and adhesion of platelets (Asselin, et al., 1997). Dianthins E and 4-methoxydianthramide were isolated from Chinese medicinal plant *Dianthus superbus* and have shown cytotoxic activity against human hepatocellular carcinoma Hep G2 cancer cell lines with IC_{50} of 2.37 and 4.08 μ g/mL. These studies were conducted concurrently with doxorubicin, an anticancer drug with an IC_{50} of 0.19 μ g/mL (Hsieh, et al., 2004). Segetalins A-H were isolated from the seed of *Vaccaria segetalis*,

which has been used as a traditional Chinese medical treatment for regulation of menstrual disturbance, stimulating blood circulation and promoting milk secretion (Huang, 1993; Morita, et al., 2006; Morita, et al., 1994; Morita, et al., 1996; Morita, et al., 1995; Yun, et al., 1997). Segetalins A, B, G and H have shown estrogen activity by increase in uterine weight in ovariectomized female rats. It has been proposed that the sequence of Trp-Ala-Gly-Val in segetalins A, B and G and Phe-Ser-Gly in segetalin H might be responsible for estrogen activity by comparing the biological activity of homologous cyclic peptides (Morita, et al., 1995; Yun, et al., 1997). Segetalin E has shown moderate cytotoxic activity against P388 leukaemia cell lines with an IC_{50} 40 μ g/mL (Morita, et al., 1996). Segetalins F, G and H containing basic amino acids such as arginine and lysine show potent vasorelaxant activity against rat aorta induced norepinephrine. In contrast, Segetalin B with no basic amino acid residues have shown estrogen like activity in ovariectomized rats with a increase in the uterus weight and the mode of action of these peptides are under study (Morita, et al., 2006). Dichotomins A–K were isolated from the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge. and the roots were used for treatment of fever in folk medicine (Morita, et al., 2005; Morita, et al., 1996; Morita, et al., 1997). Dichotomins A, B, C, E, H and I have shown cytotoxic activity against P388 leukaemia cell lines with an IC_{50} of 2.5 μ g/mL, 3.5 μ g/mL, 5.0 μ g/mL, 2.0 μ g/mL, 3.0 μ g/mL and 2.3 μ g/mL respectively. The first three cyclic peptides differ with only one amino acid residue present at 5th position in the cyclic sequence (dichotomin A: Val, B: Thr, C: Ala). In addition, the sequence of Tyr-Ala-Phe in dichotomin E is similar to dichotomins A, B and C (Phe-Leu-Tyr) in which a hydrophobic amino acid is located between two aromatic amino acids. Dichotomins D, F and G have shown moderate cyclooxygenase inhibitory activity (72% inhibition at 100 μ m for D and F; 62.6% inhibition at 100 μ m for G) (Morita, et al., 1996; Morita, et al., 1997). Dichotomins J and K have shown moderate vasorelaxant activity on norepinephrine induced rat aorta (Morita, et al., 2005).

RA-VII is an cyclic hexapeptide isolated from *Rubia akane*, which has shown cytotoxic effects against P388 lymphocytic leukemia cells, MM2 mammary carcinoma cells and KB cells (Itokawa, et al., 1984). It has also shown antitumor activity against various tumor cell lines (Majima, et al., 1993). Astin B isolated from *Aster tartaricus* showed antitumor activity against Sarcoma 180A, (Morita, et al., 1995) and induced apoptosis in human papillary thyroid carcinoma cell lines (Cozzolino, et al., 2005) (Figure 2.3).

2.3 Chemical Modification

Cyclic peptides belong to important class of naturally occurring biologically active compounds. For example, gramicidin S (Kratzschmar, et al., 1989) and tyrocidine A (Mootz & Marahiel, 1997) produced by the bacteria *Bacillus brevis* are used as antibiotics whereas cyclosporine A (Emmel, et al., 1989) is used as an immunosuppressant drug. Cyclic peptides are more stable than linear peptides and resist protease degradation. By cyclization of the mobile ends of amino acids, they are stable towards N- and C-exopeptidases (Driggers, et al., 2008). Selective transformation of functional groups on biomolecules such as carbohydrates and peptides is important in biomedical research to prepare analogues and study structure-function relationships. However, chemical modification at a specific site is often a formidable challenge. The individual group has to be selectively activated by utilizing reactivity differences among functional groups in a compound. Modified cyclic peptides may be produced by several approaches. In a classical approach, amino acids are sequentially added to a solid phase support (Merrifield resin), and then they are released by a reaction that results in cyclization (Merrifield, 1963, 1965, 1969). A second approach requires backbone modifications of the peptide at carbonyl and amide linkages (Deska & Kazmaier, 2008). Third approach utilizes the active functional groups present in the side chain of peptides for modification. The third approach is particularly efficient in peptides that have few heteroatoms in side chains and are cyclic and thus do not need the addition of protective groups to the carboxylic and amine functionality of the peptides (Kotha & Lahiri, 2005). Bis-cysteine cyclic peptides were synthesized from covalent bonding of the two non-adjacent cysteine amino acids present in the peptide chain. These compounds were prepared under highly dilute conditions in presence of DCC as coupling reagent. (Wang, et al., 2006). A further advantage of side chain modification is realized with mild reactions that preserve the chirality of the peptides. Where this is possible, efficient modification of a parent peptide and generation of many derivative compounds may be achieved. This approach can help to generate families of analogs that are suited for studies of structure/function activity relationships of biologically active peptides (Jamonnak, et al., 2010). There are about 13 proteinogenic amino acids having hetero functional groups in their side chains available for chemical transformations.

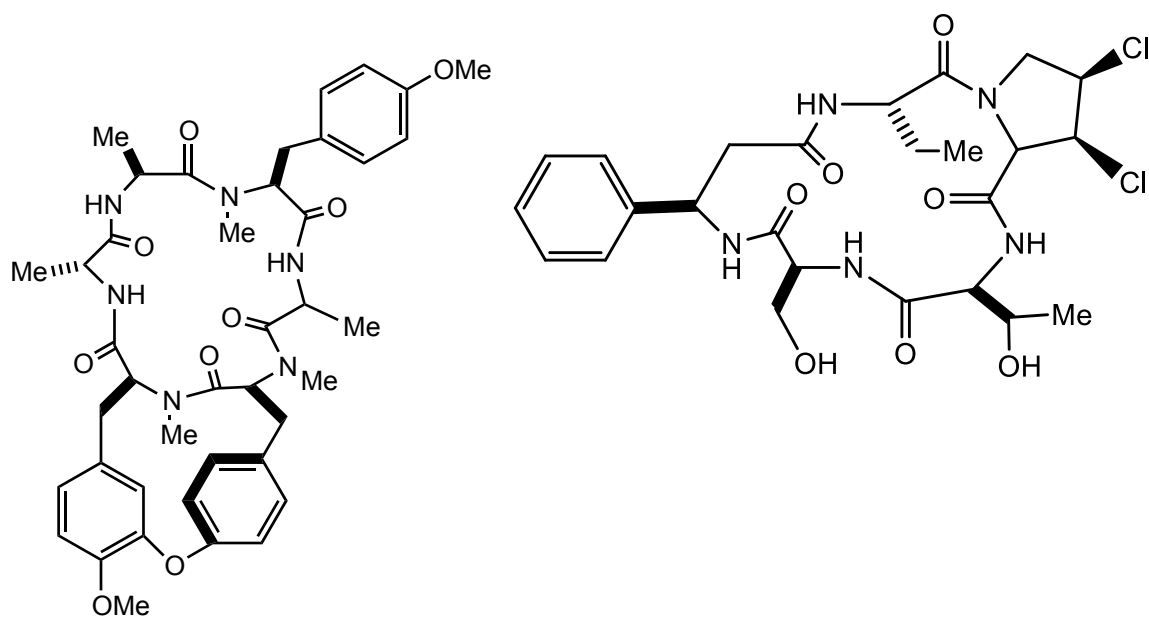
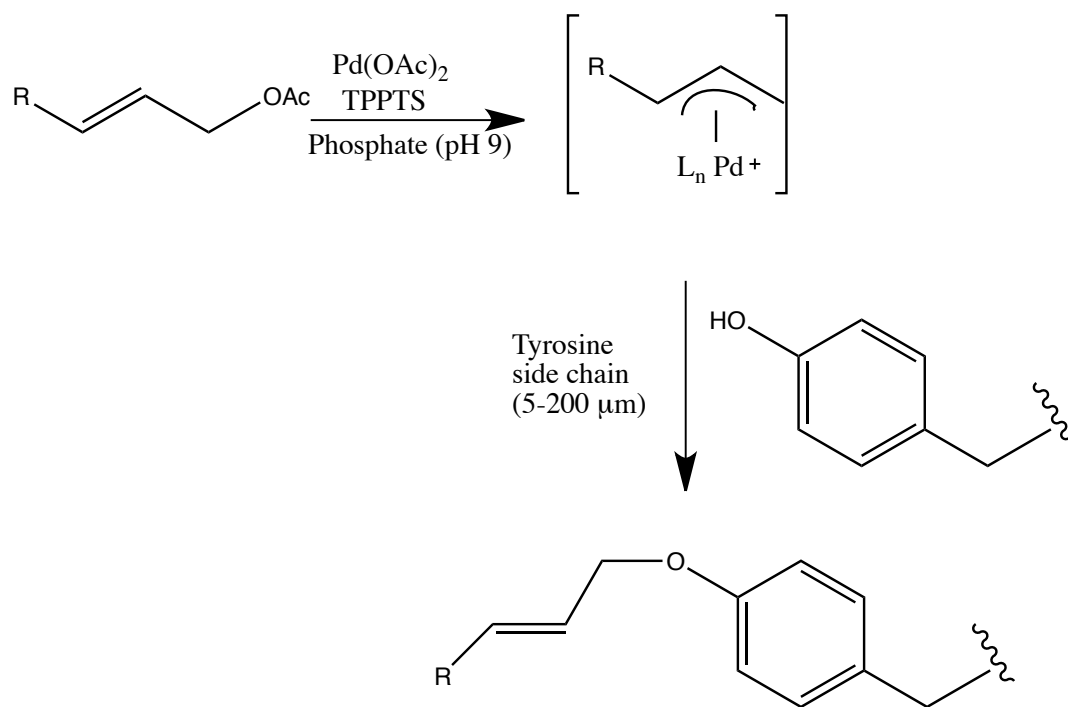


Figure 2.3 Structures of RA-VII and astin B



TPPTS- triphenylphosphine tris(sulfonate)

Figure 2.4 Reaction of tyrosine with allyl acetate

2.3.1 Chemical Modification through Active Side Chains

Peptides have been modified with various non-peptidic groups such as carbohydrates (glycopeptides or phosphopeptides), phosphoryl groups, terpenoids, polyketides etc. There are 20 standard proteinogenic amino acids present in the nature with different side chains. These side chains do not participate in the peptide bond formation and hence may be free to react. The amino acids containing active heteroatoms include lysine (-NH₂), serine (-OH), threonine (-OH), cysteine (-SH), tyrosine (-OH), tryptophan (indole group), aspartic acid (-COOH), glutamic acid (-COOH), arginine (guanidine group), histidine (imidazole group), asparagine (-CONH₂), glutamine (-CONH₂) and Met (-SCH₃) (Hermanson, 2008). Lysine is widely used for side chain modification of peptides and it readily reacts with the aldehydes or ketones, activated carboxylic acids and isothiocyanates/isocyanates (Antos & Francis, 2006). Cysteine containing sulfhydryl group reacts with various reagents such as vinyl sulfones, α -halocarbonyl compounds and maleimides to form thioether bonds (Klok, 2005). Carboxylate group of aspartate and glutamate is activated by carbodiimides to react with other functional groups to form ester, amide, thioester and hydrazide derivatives. Tyrosine amino residue can also undergo both nucleophilic and electrophilic reactions on the phenolate ion and aromatic ring respectively (Gauthier & Klok, 2008; Hermanson, 2008). Hydroxyl groups in serine have been used as a reactive centre in elimination-addition (Sommerfeld & Seebach, 1993) and metal cross-coupling reactions (Dunn, et al., 1995). This side chain approach using natural amino acids helps to synthesize various analogs. However the limitation will be to design chemoselective modification reagents for the specific amino acid in presence of other amino acids. The greater the number of amino acids in the peptide chain with the same functional group creates difficulty in synthesis where it is desirable to modify just one of the amino acids.

Non-canonical (non natural) amino acids have been incorporated in the peptide side chains by using transition metal catalysis. In these types of reactions, vinyl or aryl halides are activated by Pd (0) to form a Pd complex that reacts with specific functional groups. The special functional groups include boronic acids, organotin compounds, esters, terminal alkynes and alkene. For example, Alkylation of tyrosine residue of the protein was achieved using pi-allyl palladium complex allyl acetate (Tilley & Francis, 2006) (Figure 2.4). In addition, 1,3 dipolar cycloaddition between the azides from the side chain and alkyne forms 1,2,3-triazole derivatives using Cu (I) catalyst regioselectively under mild conditions (Tanaka, et al., 2007). Another

strategy was used to convert threonine to *allo*-threonine using oxazoline intermediates. *allo*-threonine amino acid has inversion of configuration at the β -carbon of threonine (Wipf & Miller, 1993) and it is found in biologically active peptides such as cyclodepsipeptide complex CDPC 3510 (Flippin, et al., 1989), viscosin (Burke Jr, et al., 1989) and phytotoxic syringopeptins (Ballio, et al., 1991). These amino acids were prepared by first coupling of threonine with Burgess reagent to form oxazoline intermediate and finally hydrolysis of this intermediate leads to inversion of the β -carbon atom (Wipf & Miller, 1993).

2.3.2 Chemical Modification through Methionine

The modification at the Met residue is comparatively rare as it is less reactive than the more commonly modified amino acids. Alkylation of Met residues was reported in proteins such as ribonuclease A and chymotrypsin using α -halo acetic acid and its derivatives (Gundlach, et al., 1959a; Lawson & Schramm, 1962). Iodoacetic acid was used as alkylating agents for sulfhydryl groups. This reagent can also react with ϵ -amino group of lysine, phenolic hydroxyls of tyrosine residue and the imidazole of histidine residues. However the reaction is slower at slightly acidic or neutral pH compared to sulfhydryl group (Korman & Clarke, 1956).

The reaction of Met and iodoacetic acid yields a sulfonium salt. The sulfonium salt from L-methionine was characterized by the elemental analysis and confirmed by decomposition into various products in presence of strong acid (Gundlach, et al., 1959a). It was also reported that Met can be regenerated from sulfonium salts using mercaptoethanol (Naider & Bohak, 1972). This approach was used for isolation of Met peptides under acidic conditions (Grunert, et al., 2003). The plausible mechanism for the alkylation of Met with iodoacetic acid is to first form a sulfonium intermediate (carboxymethylsulfonium salt) at 40 °C for 24 h. This sulfonium salt can be decomposed to different products depending on reaction conditions (Figure 2.5). Alkylation of L-methionine to S-carboxymethylhomocysteine occurred at 110 °C for 20 h using 6 N HCl. In addition, the sulfonium salt decomposes to homoserine at 100 °C for 1 hour in the presence of 0.2 M phosphate buffer and pH 6.5. In addition, when the sulfonium salt was heated at 100 °C for 1 hour at pH 2.2 using 0.2 M citrate buffer, homoserine lactone was formed along with homoserine (Gundlach, et al., 1959a). The rate of L-methionine alkylation in ribonuclease A is independent of pH. Met residue of ribonuclease A are efficiently alkylated at pH 2.8 and 40 °C. (Gundlach, et al., 1959a; Gundlach, et al., 1959b).

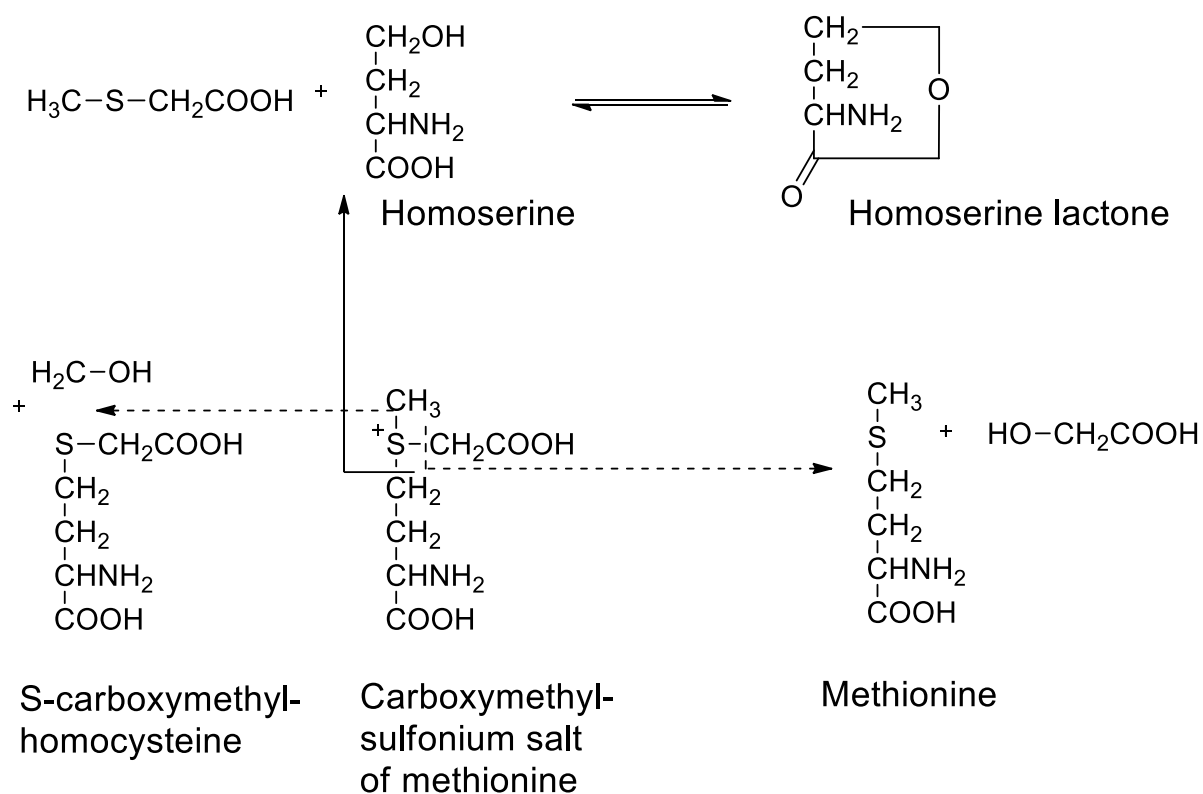


Figure 2.5 Decomposition of sulfonium salt. Adapted from Gundlach et al. (1959a)

The acidic hydrolysis of sulfonium salts of L-methionine and protein ribonuclease A leads to different products. After hydrolysis, Met, S-carboxymethylhomocysteine and homoserine (and its lactone) products were formed (Gundlach, et al., 1959a; Gundlach, et al., 1959b).

2.4 Flax Cyclolinopeptides

Flaxseed oil contains natural hydrophobic cyclic peptides (cyclolinopeptides/CLs) comprising eight or nine amino acid residues (Figure 2.6, Table 2.3). The first discovered CL, and indeed first discovered plant CL **1** was isolated in 1959 (Kaufmann & Tobschirbel, 1959). Since then, eight other CLs (**2–9**) were isolated and detected from flaxseed and characterized by NMR and MS methods (Brühl, et al., 2007; Matsumoto, et al., 2001; Morita, et al., 1999; Morita, et al., 1997). In addition, there are two CLs **10** and **11** were also chemically synthesized from parent CLs **5** and **3**, respectively (Matsumoto, et al., 2001). However, these compounds were also detected in heat treated flax meal and additionally obtained from silica that has been in contact with flax oil (Jadhav, et al., 2013).

Crystal structures of CLs **1**, **2** and **11** have been reported (Di Blasio, et al., 1989; Jadhav, et al., 2011; Matsumoto, et al., 2002; Quail, et al., 2009; Schatte, et al., 2012). However, another CL containing the non-proteinaceous amino acid *N*-methyl-4-aminoproline was isolated from linseed mill cake (Picur, et al., 1998). CLs were previously numbered from prolyl residue as the first amino acid in the sequence based on mass spectrometric fragmentation (Stefanowicz, 2004). Recently published flaxseed DNA sequences such as gene cyclolinopeptide-51 showed the embedded sequences of **1** (ILVPPFFLI), **2** (MLIPPFVI) and **5** (MLVFPLFVI) and gene cyclolinopeptide-65 showed the embedded sequences of **4** (MLLPFFWI), **6** (MLMPFFWV) and **7** (MLMPFFWI). These DNA sequences have revealed that isoleucine (Ile) is the first amino acid residue in **1**, whereas Met is the first amino acid residue in sequences of the remaining CLs (Table 2.3) (Covello, et al., 2012; Gui, et al., 2012).

2.4.1 Biological Activity of Cyclolinopeptides

Kessler and coworkers reported the first biological activity of **1** and they have shown that it inhibits cholate uptake into hepatocytes (50% inhibition at 3 μ m). This inhibitory property of **1** might prevent phalloidin intoxicification of hepatocytes by inhibiting uptake of bile acids. It has been suggested that the tripeptide Pro-Phe-Phe fragment present in **1** is similar to bioactive fragment in somastostatin and antanamide responsible for this activity (Kessler, et al., 1986).

Table 2.3 The amino acid sequences of CLs (1–15).

CL (number)	Old name	Amino acid sequence (<i>cyclo</i> -)	Molecular formula	[M+H] ⁺
A (1)	CLA	Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile	C ₅₇ H ₈₅ N ₉ O ₉	1040.6900
1-Met-CLB (2)	CLB	Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	C ₅₆ H ₈₃ N ₉ O ₉ S	1058.6461
1-Mso-CLB (3)	CLC	Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	C ₅₆ H ₈₃ N ₉ O ₁₀ S	1074.6382
1-Mso-CLD (4)	CLD	Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile	C ₅₇ H ₇₇ N ₉ O ₉ S	1064.6001
1-Mso-CLE (5)	CLE	Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile	C ₅₁ H ₇₆ N ₈ O ₉ S	977.5822
1-Mso,3-Mso-CLF (6)	CLF	Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val	C ₅₅ H ₇₃ N ₉ O ₁₀ S ₂	1084.5359
1-Mso,3-Mso-CLG (7)	CLG	Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile	C ₅₆ H ₇₅ N ₉ O ₁₀ S ₂	1098.5535
1-Mso,3-Met-CLG (8)	CLH	Mso-Leu-Met-Pro-Phe-Phe-Trp-Ile	C ₅₆ H ₇₅ N ₉ O ₉ S ₂	1082.5236
1-Met,3-Mso-CLF (9)	CLI	Met-Leu-Mso-Pro-Phe-Phe-Trp-Val	C ₅₅ H ₇₃ N ₉ O ₉ S ₂	1068.5534
1-Msn-CLE (10)	CLJ	Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile	C ₅₁ H ₇₆ N ₈ O ₁₀ S	993.5826
1-Msn-CLB (11)	CLK	Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	C ₅₆ H ₈₃ N ₉ O ₁₁ S	1090.6395
1-Met-CLD (12)	CLD'	Met-Leu-Leu-Pro-Phe-Phe-Trp-Ile	C ₅₇ H ₇₇ N ₉ O ₈ S	1048.6048
1-Met-CLE (13)	CLE'	Met-Leu-Val-Phe-Pro-Leu-Phe-Ile	C ₅₁ H ₇₆ N ₈ O ₈ S	961.5904
1-Met-CLF (14)	CLF'	Met-Leu-Met-Pro-Phe-Phe-Trp-Val	C ₅₅ H ₇₃ N ₉ O ₈ S ₂	1052.5132
1-Met-CLG (15)	CLG'	Met-Leu-Met-Pro-Phe-Phe-Trp-Ile	C ₅₆ H ₇₅ N ₉ O ₈ S ₂	1066.5627

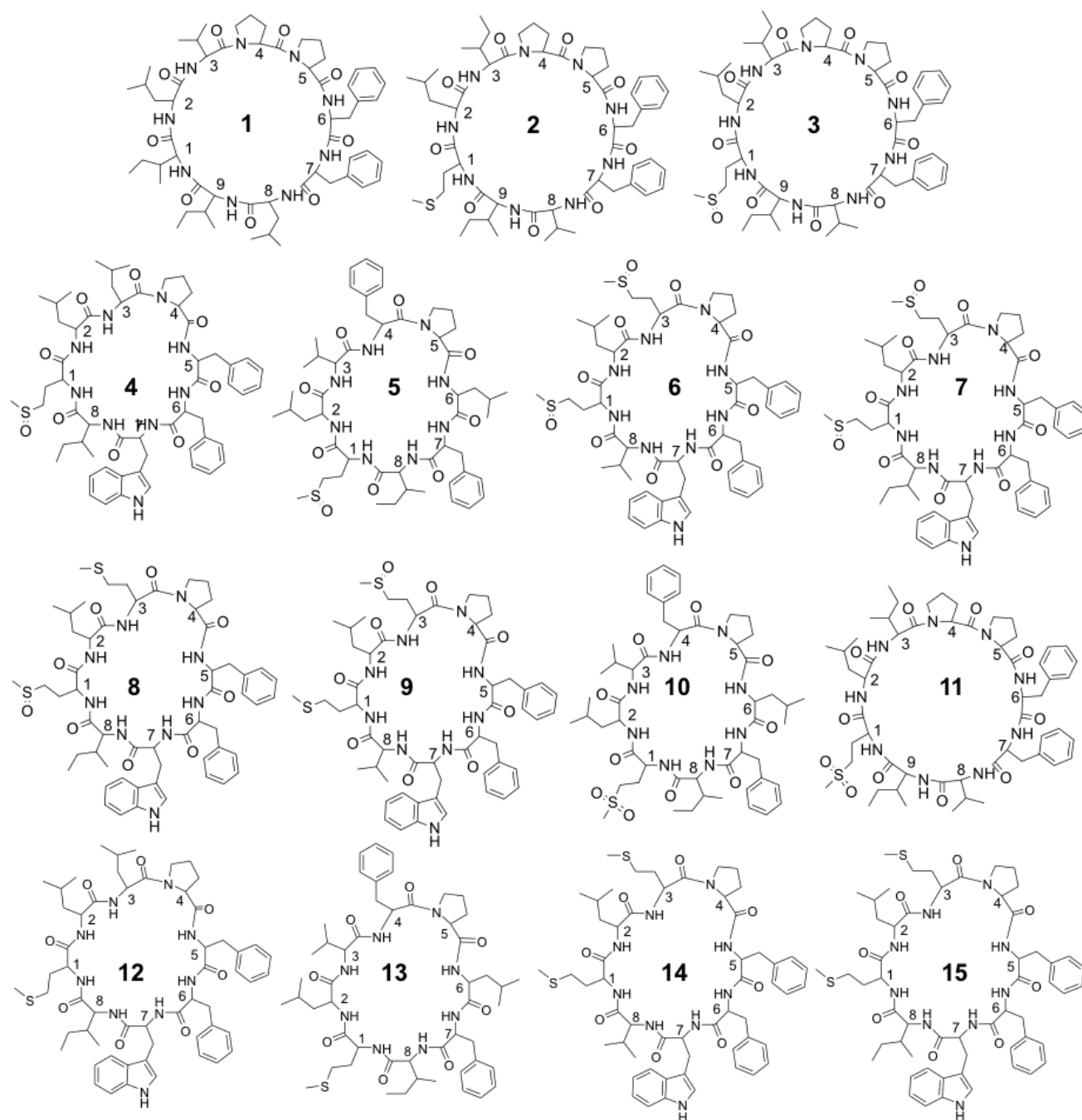


Figure 2.6 Structures of CLs (1–15)

1 has also shown immunosuppressant activity comparable to cyclosporine A. The effect of **1** on the primary and secondary humoral response was studied by plaque forming cell tests (PFC) in both *in vitro* and *in vivo*. Based on PFC, **1** inhibits both interleukin-1 α and interleukin-2. In addition, the cellular immune response in mice by **1** was determined using skin-allograft rejection, graft-vs-host reaction and delayed-type hypersensitivity. **1** influences the proliferation of human lymphocytes *in vitro* and alleviates the post-adjuvant polyarthritis in rats and hemolytic anaemia of New Zealand Black mice and the studies on these animals have shown little toxicity at a dose of 230 mg/Kg, i.v. (Wieczorek, et al., 1991). Gorski group also showed that **1** suppresses the activity of phosphatase in T cell activation to inhibit the activation and proliferation of T-lymphocyte (Gorski, et al., 2001). The molecular mechanism of **1** and CsA is reported to be similar, i.e. first they complex with cyclophilin, interact with the calcineurin system and inhibiting its phosphatase activity (Siemion, et al., 1999). Several analogs of **1** were synthesized and tested for immunosuppressive activity, but they have shown lower activity (Siemion, et al., 1999).

CL **1** and its analogs have also shown antimalarial activity. It has been proposed that strong hydrophobicity of these CLs helps in the inhibition of human malarial parasite *Plasmodium falciparum* in culture and the influence of **1** on cell membranes might lead to antimalarial activity. The substitution of L-Phe to D-Phe produces a CL that has less activity (Bell, et al., 2000).

Other CLs have exhibited different biological activity. CLs **2** and **5** inhibit the proliferation of mouse lymphocytes induced by concanavalin A with an IC₅₀ of 39 μ g/mL and 43 μ g/mL, respectively (Morita, et al., 1999). Reaney et al. have shown anticancer activity of CLs where CLs **1**, **3** and **5** induces apoptosis in a human lung epithelial cancer cell line (Reaney, et al., 2013). A recent review described two new potent immunosuppressant CLs **10** and **11**, which had an inhibitory effect on concanavalin A with an IC₅₀ of 28.1 μ g/mL and 25.2 μ g/mL respectively (Morita & Takeya, 2010).

2.4.2 Isolation and Detection

Isolation and purification of CLs is as a prerequisite to performing meaningful biological assays (Reaney, et al., 2013). Various groups have isolated CLs from flax such as, Kaufman et al. showed the recovery of CLs from precipitated slime, obtained from flaxseed processing

(Kaufmann & Tobschirbel, 1959), while Morita et al. showed the recovery of CLs (**2–9**) from the methanolic extracts of flaxseed, press cake and roots (Matsumoto, et al., 2001; Morita, et al., 1999). The latter method involves extraction of CLs using hot methanol, removal of solvent followed by column chromatography using diaion HP-20 media. The methanolic fractions were subjected to silica gel column fractionation using a CHCl₃: MeOH gradient to obtain small quantities of these CLs (recovery-0.007% of **1**, 0.0002% of **2**, 0.0037% of **3**, 0.0015% of **4**, 0.0058% of **5**, 0.0008% of **6**, 0.0024% of **7**, 0.0002% of **8** and 0.00007% of **9** from dry roots of flax) (Matsumoto, et al., 2001; Morita, et al., 1999). These CLs were characterized using two dimensional NMR, ¹H-NMR, ¹³C-NMR, IR and HR-FABMS. Stefanowicz (2001) extracted CLs from ground flaxseed (5 g) using acetone (100 mL) overnight. After removal of acetone the residue was dissolved in methanol and hydrolyzed with 10% sodium hydroxide. The solvent was removed and CLs were extracted using ethyl acetate. This protocol has lead to the recovery of CLs with Met in its reduced form along with other CLs. These reduced CLs contains Met in place of Mso and includes **12**, **13**, **14** and **15** characterized using tandem MS fragmentation patterns (Stefanowicz, 2001). But this process does not allow isolation and separation of all the CLs. Reaney et al. (2013) have developed a method to recover all reported CLs using a solid phase extraction process. The method involves extraction of oil from plant material and use of silica gel chromatography to separate CLs from oil. This method allows the recovery of 146 mg of crude CLs from 100 g of flaxseed oil (0.146% of starting material) (Reaney, et al., 2013). The recovery of CLs from flax oil is less laborious and potentially much less expensive using this concentrate.

2.4.3 Chemical Synthesis

Another approach to obtain CLs is through chemical synthesis. Various analogs of CLs were synthesized to study structure activity relationship studies. For example, Wiezorek et al. described synthetic preparation of **1** using Merrifield resin. This process requires protection of each amino acid with t-butoxycarbonyl group before coupling (Wiezorek, et al., 1991). Previously various analogs of **1** were synthesized by changing amino acid residues to form both cyclic and linear analogues. Some of the examples include substitution of each amino acid with alanine (a method called alanine scanning) to form linear analogs. The other examples include exchange of Phe with PheSO₃Na, D-tyrosine, tyrosine, D-tryptophan and tryptophan and

substitution of all the aliphatic amino acids with threonine to form both linear and cyclic analogs (Siemion, et al., 1999). Zabrocki et al. have synthesized various analogs of **1** and **2** to perform structure activity relationship studies. The two dipeptide segments (Val-Pro and Pro-Pro) of **1** were substituted with tetrazole derivatives to form both linear and cyclic analogs using solid phase peptide synthesis and linear chains were cyclized using O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) reagent (Kaczmarek, et al., 2002). In another example, α -hydroxymethyl-leucine was used for substitution of Leu and α -hydroxymethylvaline for Val residue in **1** (Zubrzak, et al., 2005) Analogs of **1** were also synthesized by replacing Pro with β -isoPro and β -homoPro and substitution of Phe with β^3 -Phe, homoPhe and *N*-benzylglycine to perform structure-activity relationship studies (Drygala, et al., 2009; Kaczmarek, et al., 2009; Katarzyńska, et al., 2008; Zubrzak, et al., 2004). In addition, the dipeptide fragment of Phe-Phe was also replaced with D-Phe-D-Phe and Phe-Phe containing ethylene bridge between nitrogens of adjacent Phe residues (Katarzynska, et al., 2011). An analog of **2** was synthesized using solid phase synthesis where the Met residue was substituted with α -hydroxymethyl methionine (Witkowska, et al., 2004).

2.5 Potential Applications of Cyclic Peptides

Peptides have found various applications in different fields including biomedical applications, biomaterials, fluorescence labeling, protein purification, antibody production and many more (Cai, et al., 2008; Fairman & Akerfeldt, 2005; Kemmer, et al., 1997; Manea, et al., 2008; Santos, et al., 2012). Peptides have self-assembled and self organized structure with non covalent interactions such as hydrophobic, van der Waals, hydrogen bonding, aromatic pi-stacking, electrostatic and metal-ligand interactions. These features help them in binding to receptors and their use in medical applications. The biomedical applications ranges from drugs, drug delivery agents to active ingredient in nutraceuticals and functional foods (Santos, et al., 2012). They are also used as biomaterials such as hydrogels, filaments and fibrils, surfactants, peptide hybrids and nanomaterial (Fairman & Akerfeldt, 2005).

2.5.1 Fluorescent Labels

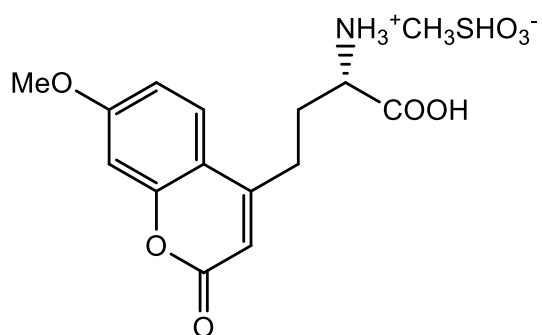
Fluorescent labels such as dyes, quantum dots and green fluorescent proteins are used to study the dynamic behaviour of compounds. Fluorescent labels may be categorized based on the fluorophore chemistry. Fluorophores may be organic compounds (e.g. coumarins, squarylium

dye, cyanine dye, fluorescein, Figure 2.7); inorganic particles (such as quantum dots) and fluorescent proteins (green fluorescent proteins and their mutants) (Katritzky & Narindoshvili, 2009). A protein of interest may be labeled by selective chemical or enzyme mediated synthetic chemistry. For example, -COOH group present in coumarins, fluoresceins and squarylium dye was used to attach the molecule of interest. For example, the peptide Cys-Leu-Ser-Tyr-Tyr-Pro-Ser-Tyr-Cys was labeled with fluorescein for *in vitro* optical imaging (Katritzky & Narindoshvili, 2009; Lee, et al., 2010; Thapa, et al., 2008). Tagging genes with genes encoding fluorescent proteins is possible through genetic engineering (Katritzky & Narindoshvili, 2009). The molecules tagged with fluorescent labels provide a mechanism for tracking, quantifying, and visualizing molecular distribution and movement of molecules in living cells. The interaction of a compound of interest with complex biological systems is possible with fluorescent labels by providing both temporal and spatial resolution of cellular and organ events. Labels may be used both *in vitro* and *in vivo* to monitor the movement of compounds and their interactions with molecules and structures of living cells (Lee, et al., 2010).

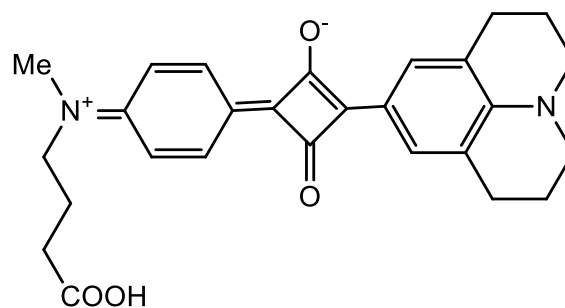
The selection of fluorescence labels depends on the *in vitro* and *in vivo* applications. For example, for *in vitro* studies, fluorophores such as fluorescein isothiocyanate, 7-amino-4-methyl coumarin and 5-carboxytetramethylrhodamine with the wavelengths between 400 and 600 nm are suitable. However, for *in vivo* imaging, fluorophores with higher wavelength range between 650 and 900 nm such as polymethines and heptamethine cyanines are often preferred. The photons emitted by higher wavelength fluorophores interfere less with the background, which may contain hemoglobin, water and deoxyhemoglobin, and penetrates into the tissues more efficiently than photons produced by fluorophores that emit lower wavelengths (Ferro-Flores, et al., 2010; Lee, et al., 2010; Weissleder & Ntziachristos, 2003).

2.5.2 Fluorescence Labeled Peptides

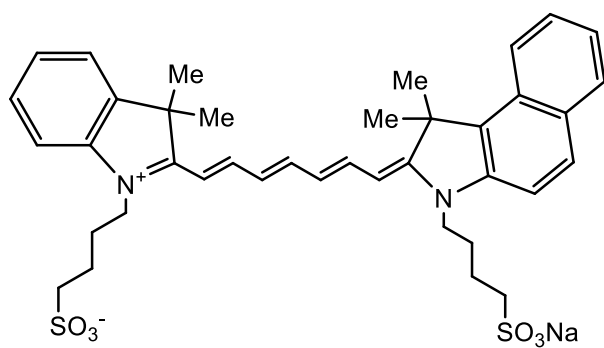
Peptides can be labeled with fluorophores for optical imaging. They have an added advantage over proteins and antibodies because of their inherent small structure. They often are less toxic than antibodies while having good permeability and low immunogenicity. Naturally occurring linear peptides are subject to tissue and blood proteases and peptidase and thus have low metabolic stability (Ferro-Flores, et al., 2010).



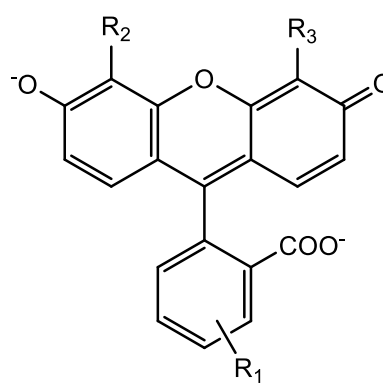
Coumarins



Squarylium dye



Cyanine dye



Fluorescein

Figure 2.7 Structures of fluorescent dyes

The stability of linear peptides may be increased by incorporation of unusual amino acids, D-amino acids, amino alcohols, N-terminal alkyl groups or by cyclization (Ferro-Flores, et al., 2010; Lee, et al., 2010). Biologically active peptides play a regulatory role in the body and perform their function through receptor binding. Such receptors and peptides have been selected as molecular targets for optical imaging. For example, receptors of integrin, gastrin releasing peptide, somastostatin, glucagon-like-peptide-1 and alpha-melanocyte stimulating hormone have been identified for tumor receptor imaging and specific receptor binding peptides (Cai, et al., 2008; Drygała, et al., 2009; Korner, et al., 2007; Miao & Quinn, 2007; Reubi, 2003, 2007). To understand their mechanism of action through structure function analysis these specific peptides may be obtained from combinatorial libraries and coupled with fluorophores (Aina, et al., 2007).

An example of fluorescent-labeled peptide includes peptide-dye conjugate [arginine-glycine-aspartic acid (RGD)-Cy5.5], which has shown affinity to integrin receptor as contrast agent *in vitro*, *in vivo*, and *ex vivo* (Figure 2.8). Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were significantly expressed in tumor cells as compared to normal cells. Hence dye conjugated to receptor peptides were used in optical imaging for visualization and quantification of integrin levels in tumors. Cyanine dye has an absorbance maximum at 675 nm and emission maximum at 694 nm. The ϵ -amino group of lysine residue present in RGDyK peptide was coupled with N-hydroxysuccinimide ester of cyanine 5.5 dye to use as a fluorescent probe to detect U87MG glioblastoma xenograft with high contrast (Chen, et al., 2004). In another example, Hsuing et al. identified the peptides from the phage display libraries that specifically bind to dysplastic colonic mucosa. This peptide, Val-Arg-Pro-Met-Pro-Leu-Gln was labeled with fluorescein by conjugation at N-terminal of peptide using aminohexanoic acid linker. Peptide dye conjugates were monitored *in vivo* using confocal microscope and this study showed strong binding of these peptides with dysplastic colonocytes as compared to normal mucosa. It has been proposed that this conjugate will be useful for detection of early-stage colorectal cancer (Hsiung, et al., 2008).

Peptides were also used in determining the proteolytic activity of enzymes by using fluorescence resonance energy transfer (FRET) technology. In this process two fluorophores (donor and acceptor) were attached to the either side of the peptide chain. When the peptide is intact, the donor molecule transfers the non-radiative energy to the acceptor molecule and shows low fluorescence. But during the proteolysis peptide chain breaks down and leads to high fluorescence of the donor molecule. FRET peptide are sensitive tool for rapid determination of

enzymatic activity on the surface of cells in culture, crude extracts or biological fluids. For example, the enzymatic activity of prolyl oligopeptidases was determined using FRET peptide Abz-GFSPFRQ-EDDnp, where Abz acts as donor and EDDnp acts as acceptor (Figure 2.9). This enzyme cleaves at the P-F peptide bond.

The biological activity of CLs makes them good candidates for fluorescent tag labeling. CLs contain intrinsic fluorophores such as Phe and Trp in their cyclic structure. CL **1** has been reported to fluorescence energy transfer to Tb^{3+} through Phe (Chatterji, et al., 1987). However these amino acid fluorophores have limitations in terms of high background absorbance of both the excitation and emission wavelength, which would make detection of fluorescent signal in living tissues difficult. Attachment of a fluorescent tag that adsorbs and emits light at wavelengths where there is little background interference is desirable. Nevertheless there are no prior reports of the attachment of fluorescent dye to CLs. CLs contain amino acids such as Trp and Met that may be suited for chemical modification to attach of fluorescent dye. Hence CLs tagged fluorescent label can potentially be used in therapeutic monitoring and fluorescence energy transfer.

2.5.2 Protein Purification using Affinity Chromatography

Affinity chromatography is a liquid chromatographic technique, which uses strong and specific non-covalent interactions (or affinity) between a stationary phase and solutes to achieve a separation. Affinity chromatography includes all kinds of adsorption chromatography, covalent chromatography, immobilized metal-ion affinity chromatography, hydrophobic interaction chromatography, and so on (Batista-viera, 2011). For instance, in covalent chromatography the protein of interest is covalently bound to the matrix such as thiol proteases bind to thiol derivatives of agarose. Hydrophobic interactions between the hydrophobic groups of protein and matrix are utilized in hydrophobic interaction chromatography. In addition metal ions are also immobilized on matrix for purification of biomolecules. Affinity chromatography relies on the principle of molecular recognition for selective separation of molecules. Biomolecules attached to chromatographic matrix interact with highly specific affinity to those in solution (López De Alda & Barceló, 2001; Rhemrev-Boom, et al., 2001). Therefore, there is little binding of non-target molecules. Affinity chromatography is used in purification of different molecules such as nucleotides, (Sander, et al., 1966) antibodies and enzymes (Silman & Katchalski, 1966).

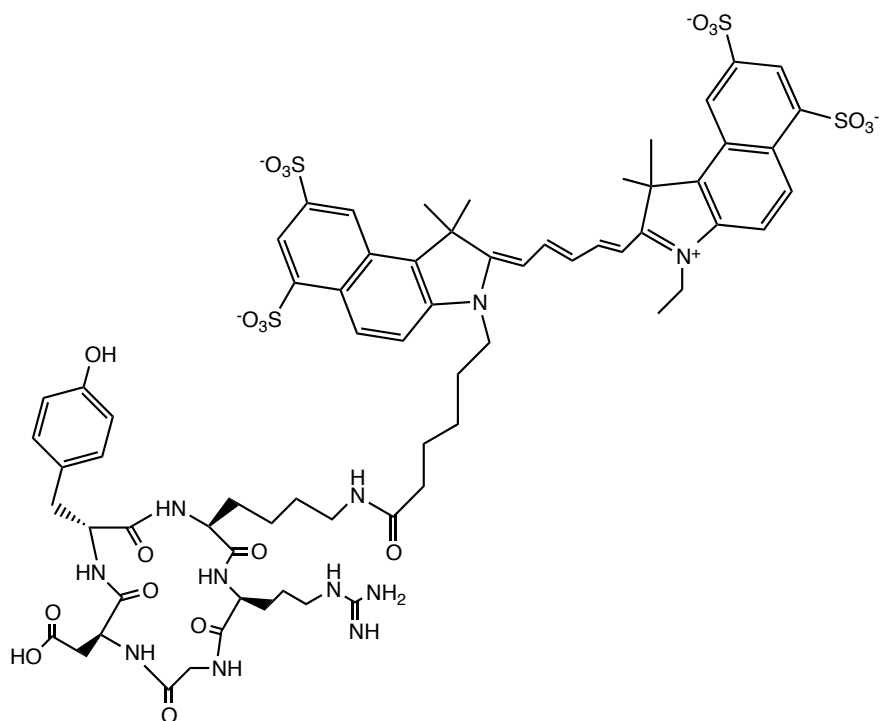
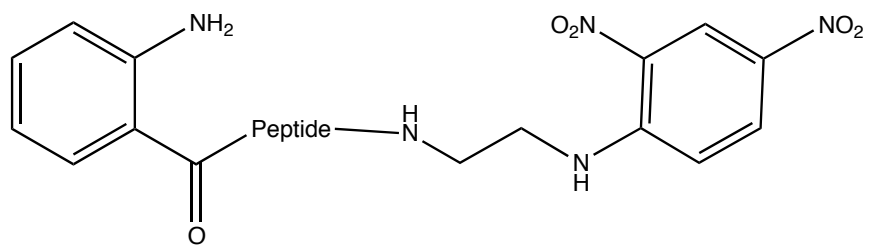


Figure 2.8 Fluorescent labelled peptide RGD-Cy5.5



Abz:
O-aminobenzoyl
(donor)

EDDnp:
ethylene diamine 2,4-dinitrophenyl
(acceptor)

Peptide- GFSPFRQ

Figure 2.9 FRET peptide Abz-GFSPFRQ-EDDnp

2.5.2.1 Affinity Media

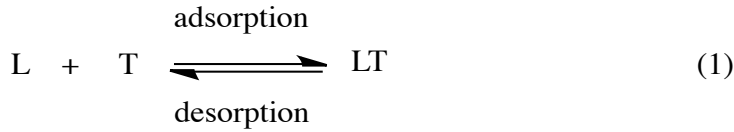
Some of the important characteristics of affinity media are: availability of simple activation methods for addition of ligands, chemical stability to the conditions of ligand addition and subsequent chromatography, low resistance to flow of biomolecules and structure that allows ready diffusion of biomolecules, high surface area that is accessible to ligand and stable to solvents. Chromatography media are divided into two groups: single composition matrices (for example- agarose, cellulose, glass) and second generation matrices (for example agarose coated polyacrylamide, glycidoxo-coated glass) (Figure 2.10) (Scouten, 1981). Agarose gels are made up of alternating residues of D-galactose and 3-anhydrogalactose, while these agarose gels are also crosslinked with polyacrylamide to form second-generation matrices. Similarly controlled pore glass is used as an inorganic matrix for immobilization and this glass can be derivatized using triethoxy propyl glycidoxysilane. These affinity media are functionalized with various activated groups. For example, agarose beads have many hydroxyl groups and polyacrylamide is functionalized with primary amides. When using these matrices as affinity media, hydroxyl groups are activated to attach spacers that can be used to attach a ligand (Varilova, et al., 2006).

2.5.2.2 Ligands

Ligands should interact reversibly and selectively with the molecule of interest. It is possible to predict potential binding site of a protein and produce a selective ligand for affinity to that protein. For example an anthraquinone-biomimetic dye-ligand was developed for L-lactate dehydrogenase binding (Labrou, et al., 1999). An example of an affinity ligand is folic acid, which contains -COOH functional groups that can easily be attached to amine containing media using carbodiimide process. This coupled folate is used for binding proteins such as dihydrofolate reductase (Kaufman & Pierce, 1971). In addition, biotin was also used to purify avidin (protein from egg white) using agarose column (Figure 2.11) (Cuatrecasas & Wilchek, 1968). It is also possible to select ligands based on functional groups (Labrou, 2003). For example, analogs of phenyl boronic acid are used as ligands for glycoproteins (Labrou & Clonis, 1994) and iminodiacetic acid is used as a ligand for metal binding proteins and metalloproteases (Clonis, et al., 1987). In the last 10–15 years, combinatorial approaches have been used in ligand selection, where libraries of compounds were constructed and subsequently used in affinity studies (Gaskin, et al., 2001). Many researchers have developed libraries from polypeptides,

polynucleotides and substituted triazines using combinatorial methods (MacLennan, 1995; Romig, et al., 1999).

The general reaction in the affinity chromatography is between the ligand (L) and target (T) to form ligand-target complex (LT) as shown in equation 1, where [L] and [T] represents ligand and target concentrations at equilibrium. Hence the dissociation constant K_D can be expressed in equation 2. The ratio of bound target to total bound is shown in equation 3, where L_0 is the concentration of the ligand in moles per volume “v”. Here v represents the volume of solution entrapped with in the pores. The ratio of bound to total must be near 1 to achieve efficient binding and therefore K_D should be smaller than L_0 . The parameter such as temperature, ionic strength and pH affects K_D and they can be altered for binding and elution of target from the ligand. In the forward reaction (adsorption) of equation 1, K_D ranges between 10^{-6} to 10^{-4} M and represents more binding and less elution. However, in reverse reaction (desorption) K_D ranges between 10^{-2} to 10^{-1} M and represents less binding and more elution (Magdeldin & Moser, 2012).



$$K_D = \frac{[L] * [T]}{[LT]} \quad (2)$$

$$\frac{\text{Bound target}}{\text{Total bound}} \approx \frac{L_0}{K_D} + L_0 \quad (3)$$

$$\text{Bound target} = [LT]$$

$$\text{Total bound} = [LT] + [T]$$

2.5.2.3 Immobilization of Ligands

The attachment of ligands to solid matrix can be performed by three methods: covalent, non covalent and coordinating. In the covalent methods, activated functional groups present in

the ligand such as amine, hydroxyl, sulfhydryl, carboxyl and aldehyde can be use as mode of attachment with the matrix (Table 2.4 and Figure 2.12). Non-covalent methods consist of non-specific or biospecific adsorption onto surface and entrapment within the pores of the matrix. In addition, ligands can also be immobilized by coordinating with metal ions in case of metal ion affinity chromatography (Kim & Hage, 2005; Magdeldin & Moser, 2012).

2.5.2.4 Peptide Affinity Tags

Peptide tags are important class of affinity ligands and they are used for immobilization, purification and detection of proteins. They have advantage over other tags like antibodies and proteins in terms of cost and stability. Peptides can be readily synthesized from amino acids and various analogs can be prepared. Sometimes they also belong to class of hydrophobic chromatography in which hydrophobic amino acid residues play important role. Activated solid matrix can be coupled with peptides containing compatible functional groups and regenerated. However, the challenge in peptide affinity chromatography is to obtain specific peptide for the protein binding. But by screening ligands from biological combinatorial libraries (phage display library) or from solid combinatorial libraries (one bead one peptide library), specific peptides can be obtained (Baumbach & Hammond, 1992; Huang & Carbonell, 1995; Tozzi, et al., 2003). The amino, hydroxyl, carboxyl groups present in the amino acids such as lysine, serine, threonine, tryptophan, aspartic acid, glutamic acid are mostly used for coupling with the solid matrix. Some of the examples of peptide ligands include purification of anti-granulocyte macrophage colony stimulating factor monoclonal antibody using tetrapeptide Ala-Pro-Ala-Arg (Camperi, et al., 2003). A hexapeptide Tyr-Ala-Asn-Lys-Gly-Tyr was used for the purification of the serine protease coagulation Factor IX from blood plasma (Buettner, et al., 1996). Trypsin was also purified from bovine pancreas using tripeptide Thr-Pro-Arg (Makriyannis & Clonis, 1997). Cyclic peptides such as somatostatin and CL analogs have also be used in the affinity purification of rat liver cell proteins (Kemmer, et al., 1997; Wenzel, et al., 1995).

Protein-protein and protein-small solute interactions are important in many biological processes. Some of the examples include hormone binding with receptors, enzymes with substrates and binding of drugs and other compounds with serum proteins (Hage, 2002). Affinity chromatography plays important role in bioseparation of biomolecules from complex mixtures.

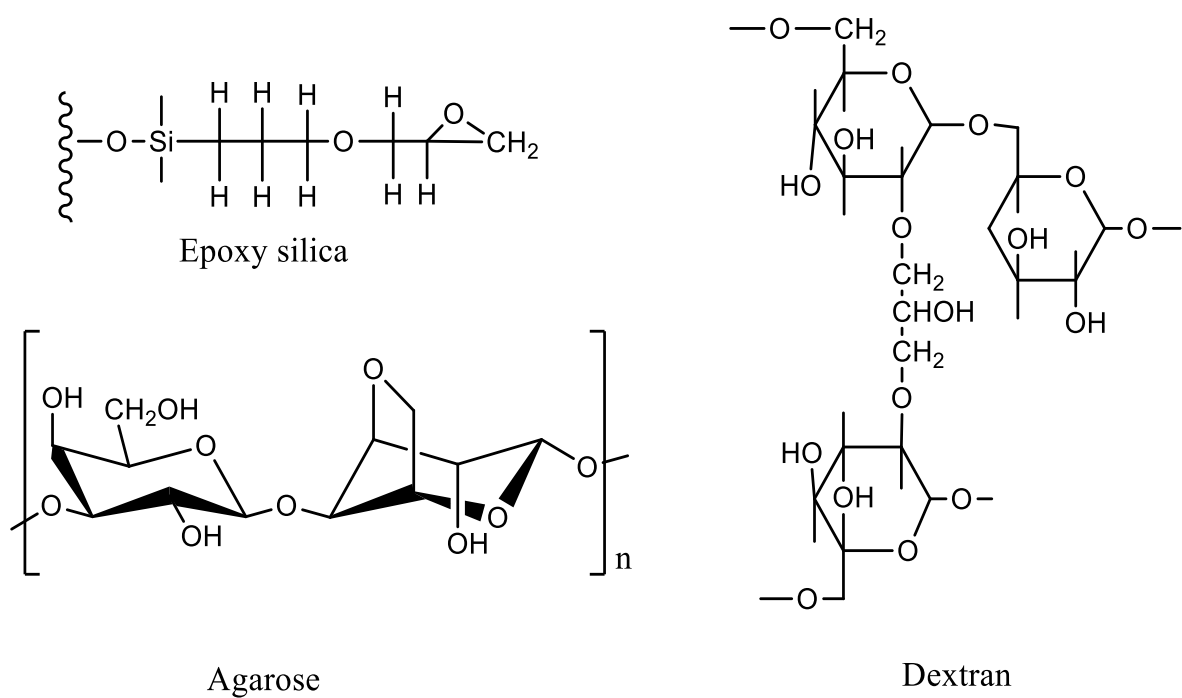


Figure 2.10 Affinity matrices

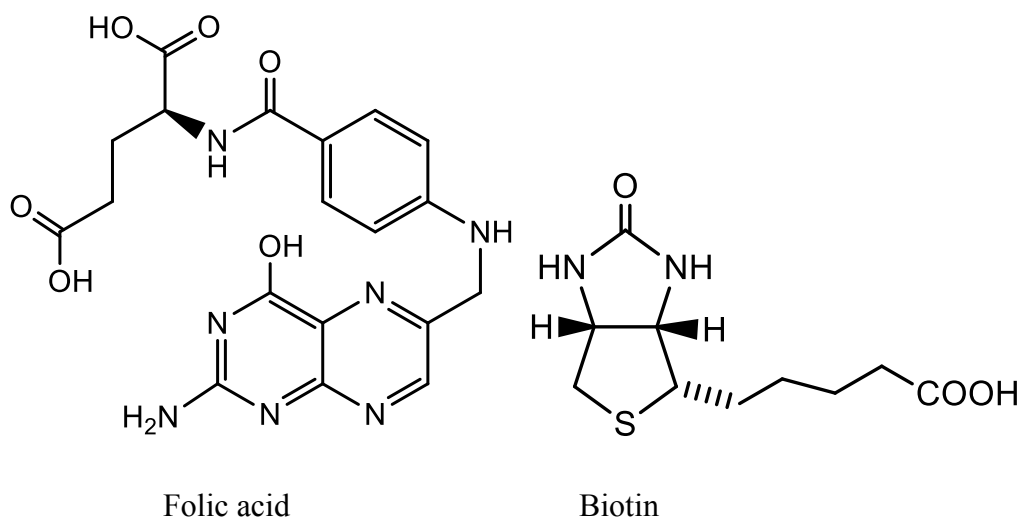


Figure 2.11 Affinity ligands

Table 2.4 Covalent Immobilization techniques (Kim & Hage, 2005).

Group	Covalent Immobilization techniques
Amine	CNBr method, Schiff base (reductive amination), <i>N</i> -hydroxysuccinimide, CDI, cyanuric chloride, azalactone method, divinyl sulfone, EDC
Sulfhydryl	azalactone method, divinyl sulfone, epoxy method, iodoacetyl/bromoacetyl, maleimide method, pyridyl sulfide, TNB-thiol
Hydroxyl	cyanuric chloride, divinyl sulfone, epoxy
Aldehyde	Hydrazide
Carboxyl	EDC

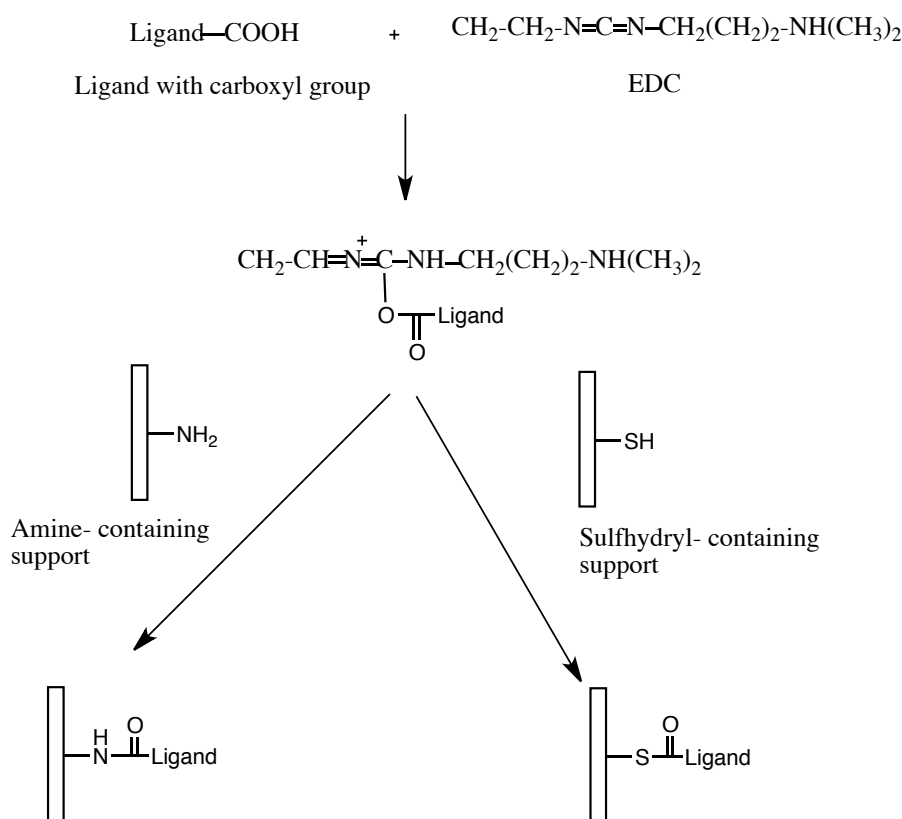


Figure 2.12 Coupling of ligand with amine and sulfhydryl containing support

It was reported that analogue of CL **1** bound to proteins in the cytosol and plasma membranes of rat hepatocytes. The structural features important for binding with proteins were hydrophobic surfaces and cyclic conformational structure of CL (Kemmer, et al., 1997).

2.5.3 Haptens for Antibody Production

Antibodies or immunoglobulins belong to special class of proteins found in extracellular fluids in vertebrates, particularly blood and lymph. They are produced by white blood cells and used as the organism's innate system for identifying and neutralizing foreign organisms such as bacteria and viruses (Singer & Doolittle, 1966). Each antibody is highly specific towards a particular compound or antigen. Antibodies consist of two kinds of polypeptide chains namely two heavy and two light chains held together in a complex by a disulfide bridge (Edelman & Benacerraf, 1962; Fleischman, et al., 1962). The structure is mostly conserved but a variable region at the tip of each antibody has binding site that has high affinity to a specific antigen. B-cells in a single vertebrate produce around 10^8 to 10^{10} unique antibodies each with different binding sites (French, et al., 1989). This innate ability of organisms to generate antibodies of specific nature has been used by researchers to produce specific antibodies for many applications including catalysis and immunodiagnostics.

It was reported that the molecule should be above 10,000 Da to illicit an immune response. Smaller molecules may be bound covalently to larger protein molecules to produce an immune response as well. A small organic molecule linked covalently to a protein in order to induce an immune response is called a hapten. When the hapten/protein complex is injected into an animal, antibodies are produced. Antibodies that specifically bind to a hapten can be studied by a number of methods including immunoprecipitation (Landsteiner, 1945). Fluorescent probes are also used to study chemical and binding properties of antibodies (Kabat, 1968; Northrup & Erickson, 1992). Examples of use of these antibodies are the detection of dioxin and pesticides using antibodies generated in response to synthetic halogenated haptens such as dibenzop-dioxin and aldicarb oxime succinic ester respectively. These haptens were conjugated with proteins such as BSA, LPH and OVA to produce polyclonal antibodies. (Figure 2.13) (Sanborn, et al., 1998; Zhang, et al., 2006).

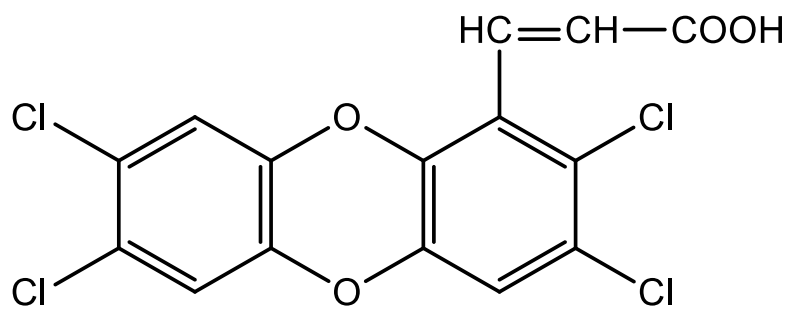
2.5.3.1 Peptide Antibodies

Peptide antibodies are used as tools in purification, identification of proteins, diagnostics,

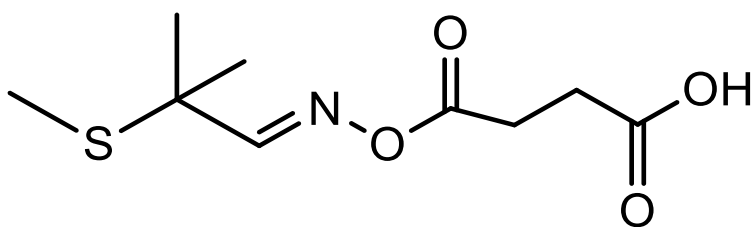
therapeutic purpose in biology. The advancement in peptide synthesis helps to generate varied sequence of amino acids and generating application specific antibodies. Peptides are conjugated with carrier proteins like ovalbumin, BSA and keyhole limpet hemocyanin to elicit antibodies (Houen, et al., 2003; Schaaper, et al., 1989; Strynadka, et al., 1988). Commonly, ϵ -amino group of lysine or the thiol group of cysteine are involved in conjugation and it was carried out by one pot and two step method (Reichlin, 1980). In the one pot method, carrier protein, cross-linking reagent and peptide are mixed together and peptide conjugate was dialyzed to remove unreacted reactants. Glutaraldehyde and carbodiimide may be used as cross-linking reagents (Goodfriend, et al., 1964; Reichlin, 1980). However, in two-step method process, carrier protein was activated with cross-linking agent and then the product reacts with the peptide. The reagents used are *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), iodoacetic acid *N*-hydroxysuccinimide ester (IAAOSu) and *N*-succinimidyl 3-(2-pyridylthio)-propionate (SPDP) (Carlsson, et al., 1978; Houen, et al., 2003; Kitagawa & Aikawa, 1976). These peptide conjugates were used to immunize various species depending on the amount of peptide and antibody required, time period response and presence of homologous protein (Trier, et al., 2012).

Peptide-based polyclonal antibodies may be used to detect a sexually transmitted pathogen, *Mycoplasma genitalium* using immunofluorescence staining (Zarei, et al., 2011). Polyclonal antibodies are also used as analytical tool as immunoblots to determine the native or denatured calreticulin in solution (Houen, et al., 1997). However, monoclonal antibodies are more specific in nature and able to detect single amino acid substitution in the peptide chain. For example, deamidated gliadin peptide (QFPQELPYPQPQ) antibody distinguishes between the amide and deamidate peptides (Skovbjerg, et al., 2004). Another example includes p53 monoclonal peptide antibody which shows specificity towards dephosphorylated peptide over mono or non-phosphorylated peptide (Otvos, et al., 1998).

CLs are bioactive molecules with molecular weight about 1 kDa and hence they can act as haptens. There are no reports on the generation of antibodies using CLs. Hydrophobic amino acids in CLs makes these CLs insoluble in water. However, CLs can be coupled to proteins such as BSA in organic solvents such as DMSO or THF to form an immunogen. The antibodies produced using these CL-BSA conjugates may be used to detect of CLs in flax and flaxseed products and for localization of CLs in living tissues.



2,3,7,8 tetrachlorodibenzop-dioxin



Aldicarboxime succinic ester

Figure 2.13 Structure of haptens linked to protein

2.6 Brief Introduction to Chapter 3

As described in Chapter 2, flax CLs are present in very small quantity (0.2%; 200 mg/100 g) in flaxseed and flaxseed products. Previously described extraction protocols were not sufficient to obtain these CLs in amounts adequate for isolation in gram quantities. The previous methods used for the recovery of CLs involve the extraction of oil from significant quantities of plant material, which do not result in large yields of CLs. The present process relates to methods for extraction, isolation and purification of individual CLs (**1**, **3**, **5**, **10** and **11**) from oil adsorbed silica. Additionally, oxidation of the enriched CL extracts was performed to simplify chromatography for recovery of specific CLs (**1**, **10** and **11**). Recovery of individual hydrophobic CLs is vital because each CL may possess different biological activity and availability of sufficient quantities will also allow for chemical modification of naturally isolated CLs.

We have found that CLs **10** and **11** are absent in commercial flax products, but they are products of ageing flax. These CLs have been fully characterized using NMR and MS methods and can be used as analytical standards. Both these CLs have shown chemical and thermal stability at high temperatures. CL **11** has been crystallized in butanol-water solvate. Table 2.3 describes the old and new nomenclatures of CLs and CLs were represented as numbers instead of names in the thesis for simplicity.

CHAPTER 3

DETECTION, ISOLATION AND CHARACTERIZATION OF CYCLOLINOPEPTIDES J AND K IN AGEING FLAX

3.1 Abstract

Methionine sulfone containing CLs **10** and **11** may be produced from their reduced forms by oxidation but it is not known if these compounds occur in food that contains flax. These compounds have been reported to possess greater immunosuppressive activity than their reduced methionine sulfoxide CLs from **3** and **5**, respectively. Since **10** and **11** have not been detected in commercial flax oil and milled flaxseed, we tested for their presence in flax food products. Here we report that **10** and **11** accumulate in ground flaxseed that is exposed to air and heat (100 °C) for more than 4 h. Standards of **10** and **11** were prepared, isolated and extensively characterized using HPLC-MS/MS, 1D and 2D NMR methods. We also report the excellent thermal and oxidative stability of these CLs. Due to the harsh conditions required to produce **10** and **11**, it is expected that their levels in flax based foods would be low and therefore their presence could serve as an indicative measure of severe oxidation of a food product.

Keywords- Cyclolinopeptides, Isolation, Characterization, Flax, Ageing

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3.2 Introduction

Flax, a member of the family Linaceae, is cultivated for production of oil seeds and fibre, and is one of the oldest crops (Jhala & Hall, 2010). The edible oil, linseed oil or flaxseed oil is obtained by extraction of flaxseed. Polyunsaturated fatty acids, lignan, mucilage and CLs are major functional classes of compounds present in flax. Consumption of flaxseed oil increases concentration of α -linolenic acid (ALA) in blood plasma (Barceló-Coblijn, et al., 2008; Kaul, et al., 2008). ALA is a precursor of the polyunsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that reduce the risk of cardiovascular diseases (Gebauer, et al., 2006). Secoisolariciresinol diglucoside (SDG) is a principal lignan in flaxseed, a phytochemical antioxidant that acts as a precursor of mammalian lignans and a phytoestrogen (Adolphe, et al., 2010; Prasad, 2004; Saggar, et al., 2010). Investigative studies on animals have shown that SDG can prevent the development of atherosclerosis and diabetes (Fukumitsu, et al., 2008; Prasad, 1999, 2009), and additional benefits include modification effects on blood lipids and cholesterol levels (Fukumitsu, et al., 2010).

Flaxseed oil contains natural hydrophobic CLs comprising eight or nine amino acid residues (Figure 2.6 and Table 2.3, Chapter 2). The first discovered linseed CL **1**, was isolated in 1959 (Kaufmann & Tobschirbel, 1959) and since then, eight other CLs **2–9** have been isolated from flaxseed oil and characterized by NMR and MS methods (Brühl, et al., 2007; Matsumoto, et al., 2001; Morita, et al., 1999; Morita, et al., 1997). Reduced CLs **12–15** (originally from **4–7**, respectively) have also been detected in flax oil and characterized using tandem MS fragmentation patterns (Stefanowicz, 2001). CLs **1**, **2** and **5** exhibit immunosuppressive activity (Gorski, et al., 2001; Morita, et al., 1999; Wieczorek, et al., 1991). In addition, CLs **1**, **3** and **5** induce apoptosis in human lung epithelial cancer cell lines (Reaney, et al., 2013) indicating that these compounds have potential applications as pharmaceuticals. CLs could be utilized as drugs or drug delivery agents as they have biological activity, hydrophobicity and may form inclusion complexes. For example, the hydrophobicity of CLs plays a significant role in transport across cell membranes in addition to distribution in tissues and organs (Kemmer, et al., 1997) and helps in binding with human serum albumin (Rempel, et al., 2010). CLs also form inclusion complexes with metal ions such as Tb (Chatterji, et al., 1987) and forms crystalline solvates with alcohols (Jadhav, et al., 2011; Quail, et al., 2009; Schatte, et al., 2012). CLs were previously numbered

from prolyl residue as the first amino acid in the sequence. Recently published flaxseed DNA sequences have revealed that isoleucine (Ile) is the first amino acid residue in **1**, whereas methionine (Met) is the first amino acid residue in sequences of the remaining CLs (Covello, et al., 2012; Gui, et al., 2012).

Cyclic peptides containing methionine sulfone (Msn), a product of Met and methionine sulfoxide (Mso) oxidation, are more rare and are not observed as natural products in higher plants (Morita & Takeya, 2010; Pomilio, et al., 2006; Tan & Zhou, 2006). These Msn containing peptides have higher thermal, acid and base stability than Met and Mso parents. Thermally stable peptides have been used in various applications such as biomedical, nanotube technologies, microporous solids and photoswitches (Afonso, et al., 2012; Cordes, et al., 2006; Ryu & Park, 2010).

A recent review described two new potent immunosuppressant CLs **10** and **11** (Figure 2.6 and Table 2.3, Chapter 2) (Morita & Takeya, 2010). Of all the eleven known CLs, **1** showed the best immunosuppressive activity, followed by **10** and **11**, which were previously synthesized in 75% isolated yield *via* oxone oxidation of **5** and **3**, respectively (Matsumoto, et al., 2001). This chapter describes detection of **10** and **11** in flax meal after accelerated ageing and preparation and isolation of **10** and **11** from a mixture of **1**, **3**, **5**, **10** and **11**. A comprehensive structure elucidation of **10** and **11** was performed using tandem mass spectrometry and, NMR (^1H , ^{13}C and 2D) methods. We also investigated the stability of these CLs when exposed to acid and base at temperature above 200 °C.

3.3 Materials and Methods

3.3.1 Materials

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. All solvents were HPLC grade and were used as such. Ground flaxseed was obtained from Bioriginal Food and Science (Saskatoon, Canada) Inc. The samples had been ground and packaged for commercial sale 1 week before the analysis was conducted. Packages were opened on the day samples were subjected to accelerated ageing at elevated heat (100 °C) under a stream of air.

3.3.2 Structural Analysis of Cyclolinopeptides

3.3.2.1 Nuclear Magnetic Resonance Methods

Proton NMR spectra were recorded on a 500 MHz Bruker Avance spectrometer (Inverse triple resonance probe, TXI, 5 mm; XWIN-NMR 3.5 software). The ^1H NMR spectra (500 MHz) chemical shifts (δ) values are reported in parts per million (ppm) relative to the internal standard TMS. The δ values are referenced to CHCl_3 in CDCl_3 at 7.27 ppm, and multiplicities are indicated by the following symbols: s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, and br = broad. For ^{13}C NMR (125.8 MHz), the chemical shift (δ) values were referenced to CDCl_3 (77.4 ppm). NMR experiments were conducted at 320 K to obtain well-resolved spectra. Fourier transform infrared (FT-IR) spectra were recorded on a Bio-Rad FTS-40 spectrometer using the diffuse reflectance method on samples dispersed in KBr (DRIFT); only diagnostic and/or intense peaks are reported. Unless otherwise noted, specific rotation $[\alpha]_D$ (methanol) was determined at ambient temperature on a Perkin-Elmer 141 polarimeter employing a 1 mL, 10 cm length; units are $10^{-1} \text{ deg}\cdot\text{cm}^2\cdot\text{g}^{-1}$ and the concentrations (c) are reported in g/100 mL.

3.3.2.2 Liquid Chromatography Methods

HPLC-DAD analysis was performed with an Agilent 1200 series HPLC system equipped with a quaternary pump, autosampler, degasser and diode array detector (Agilent G1315C/D) (wavelength range 190–300 nm). The peaks were detected at wavelengths of 214 nm with a 10 nm bandwidth and against a reference signal at 300 nm. A ZORBAX Eclipse XDB-C18 (reverse phase) column (5 μm particle size silica, 4.6×150 mm), equipped with an online filter was used for isolation of CLs from flax oil laden silica. The mobile phase consisted of a linear gradient of H_2O : acetonitrile (55:45 to 90:10 in 25 min) and a flow rate of 0.5 mL/min. Further chromatographic analyses were performed on SpeedRod Chromolith RP-18e column (2 μm particle size, 50×4.6 mm i.d.) equipped with an online filter for detection of CLs from ground flaxseed. The mobile phase consisted of H_2O -acetonitrile (70:30 to 30:70 in 4 min, 10:90 in 0.5 min, 70:30 in 0.5 min and equilibrated to 70:30 for 1 min) at a flow rate of 2 mL/min.

Preparative reverse phase chromatography was performed on a BioCAD SPRINT Perfusion Chromatography Workstation (Perspective Biosystems Inc., MA, USA) equipped with GL Sciences Inc. Inertsil Prep - octadecylsilane (ODS, 10 μm particle size silica gel, 250×30

mm) column with injection volume of 1.9 mL, and a UV/VIS detector operating at a wavelength of 214 nm. The mobile phase consisted of H₂O-acetonitrile (35:65 to 30:70 in 10 min, to 0:100 in 20 min) at a flow rate of 7.0 mL/min.

3.3.2.3 Mass Spectrometry Method

High resolution HPLC-MS was performed on an Agilent HPLC 1100 series directly connected to QSTAR XL Systems (Mass Spectrometer Hybrid Quadrupole-TOF LC-MS/MS, Applied Biosystems, Toronto, Canada). An electrospray ionisation (ESI) source was used in LC-MS/MS analysis of **10** and **11**. Chromatographic separation was achieved at room temperature using a Hypersil ODS C-18 (Hewlett-Packward, Germany, 5 µm particle size silica, 100 × 2.1 mm) column. The mobile phase consisted of a linear gradient of solvent A, H₂O containing 0.1% formic acid, and solvent B, acetonitrile containing 0.1% formic acid. The elution was performed using a linear gradient from 55% of B to 90% of B within 25 min at a flow rate of 0.25 mL/min. Further high resolution HPLC-MS-ESI analyses were performed on an Agilent HPLC 1200 series directly connected to a MicroTOF-Q II Mass Spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with electrospray ionisation (ESI) source for detection of CLs in flax meal. Chromatographic separation was achieved at room temperature using a Chromolith FastGradient RP-18e column (Merck KGaA, Darmstadt Germany, 50 × 2 mm). The mobile phase consisted of solvent A, H₂O containing 0.1% formic acid, and solvent B, acetonitrile containing 0.1% formic acid. The gradient was 2 min isocratic 40% B, followed by linear increase to 90% B in 8 min, the column was reequilibrated with the starting solvent for 6 min thereafter. A flow rate of 0.4 mL/min was used throughout each chromatogram.

3.3.3 Heat Treatment of Ground Flax Meal

The package of commercial ground flaxseed was shaken thoroughly. Samples of ground seed (3 g) were put in sample tubes of an oxidative stability index (OSI) heating block (OSI instrument, Omnion Inc., MA, USA,) and held at 100 °C and air pressure of 60 kPa. At regular time intervals, sample tubes containing ground flaxseed (120 mg) were removed from the heating block and transferred to 2 mL eppendorf tube followed by the addition of 70% (500 µL) aqueous methanol. This was then ground in a Retsch mixer mill (MM 300, Retsch GmbH, Haan, Germany) for 3 min at a vibrational frequency of 25 Hz using steel beads (5/32" SPEX cat

2150). This mixer mill offers processing of samples in 96 collection microtube racks. The tube was incubated at 60 °C for 2 h with agitation and filtered using a 0.45-µm filter.

3.4 Results and Discussion

Fresh flaxseed samples are rich in Met containing CLs **2**, **12–15** (Stefanowicz, 2001). After a period of time, Met moieties of these CLs are oxidised to Mso containing CLs **3**, **4**, **5**, **6** and **7** (Matsumoto, et al., 2001; Morita, et al., 1999). However, Msn containing CLs **10** and **11** do not occur in either fresh flaxseed extracts or in freshly pressed flaxseed oil (Section 3.4.1). These CLs were previously prepared by chemical oxidation of CLs **5** and **3** respectively (Matsumoto, et al., 2001). We therefore heated fresh ground flax to accelerate ageing and to determine if CLs **3** and **5** are produced under these circumstances. Heating and airflow led to the production of significant amounts of **10** and **11**. Thus, these CLs could serve as potential indicators of oxidative changes occurring in flaxseed products. In order to obtain sufficient CLs quantities for further biological studies, we pursued simple CL modification approaches to improve CL recovery from oil. The total concentration of CLs in flaxseed is only 0.2% making isolation of sufficient individual CLs to utilize as a standard or for analysis a laborious task (Brühl, et al., 2007; Matsumoto, et al., 2001; Morita, et al., 1999; Morita, et al., 1997). We previously reported the treatment of 2000 L of flax oil with 40 kg of silica to recover large quantities of CLs (Reaney, et al., 2013). We report here that long term adsorption of flax CLs on silica significantly alters CL composition. Tryptophan containing CLs (**4**, **6–9**) could not be recovered from silica that had been stored for 6–12 months before extraction while **3** and **5** undergo further oxidation yielding new **11** and **10**, respectively, each having Msn moieties. Since HPLC-DAD analyses of oil-laden silica extract showed presence of CLs **1**, **3**, **5**, **10** and **11**, purification of this mixture to individual constituent CLs could be time consuming due to the large number of fractions and the lower yield of each fraction. We concluded that a chemical reduction or oxidation of the crude extract would transform the CLs to similar chemical forms, that is, convert Mso CLs to corresponding Met or Msn derivatives. Reduction of the crude extract however yields CLs **1**, **3**, **5**, **10** and **11**, in which case CLs **1** and **13** co-elute (data not shown) making chromatographic separation difficult. We, therefore, resorted to oxidising the crude extract of CLs **1**, **3**, **5**, **10** and **11** to yield chromatographically well-resolved mixture of CLs **1**, **10** and **11** quantitatively. This conversion simplified the preparative chromatographic

separation from five to three CLs. Unlike previous methods (Matsumoto, et al., 2001) this also reduced the purification step of first obtaining pure CLs **3** and **5** before oxidation to access **11** and **10**.

3.4.1 Detection of CLs in Freshly Ground Flax Meal

Fresh ground flax meal was ground and extracted with 70% aqueous methanol. The samples were incubated and agitated for 2 h at 60 °C and filtered. HPLC chromatograms of the extract showed the presence of CL **1** and reduced CLs **2** and **12–15** (Stefanowicz, 2001). The structures of these CLs were confirmed using HPLC and LC-DAD trace (Figures 3.10 and 3.11). The molecular masses and retention times (parenthesis) of CLs **1**, **2** and **12–15** were determined to be 1040.6900 (4.33), 1058.6461 (4.00), 1048.6048 (4.69), 961.5904 (4.33), 1052.5132 (4.33) and 1066.5627 (4.44) respectively. CLs **1**, **13** and **14** were coeluted.

3.4.2 Detection of **10 and **11** in Accelerated Ageing**

Ground flax meal samples (3 g) were heated at 100 °C with constant air pressure of 60 kPa in the heating block of an OSI instrument. The levels of CLs were determined in triplicate samples obtained after treatment for 0, 2, 4, 8 and 16 h. CLs were extracted by the above procedure and analysed using HPLC and LC-DAD trace (Figures 3.10 and 3.12). CLs **2**, **3** and **11** are homologues of each other containing Met, Mso and Msn residues respectively. A similar pattern was also observed for CLs **13**, **5** and **10**. To display this transformation, the parent ion chromatograms were extracted from total ion mass chromatograms comparing the parent ions of **2**, **3** and **11** and separately comparing the parent ions of **13**, **5** and **10**. This extracted ion chromatographic analysis clearly showed the accumulation of **11** and **10** in the flax meal (Figures 3.1 and 3.2). The extracted ion chromatogram also shows that CLs **3** and **5** were accumulated as CLs **2** and **13** decreased in the flaxseed. Finally, after 16 h CLs **10** and **11** were also detected and their structures were confirmed by MS/MS fragmentation (Figures 3.3 and 3.4). These results show that **10** and **11** are not present in flax meal, but indicators of flax CL oxidation. We have also conducted control experiments under constant air pressure of 60 kPa at room temperature for 16 h, but formation of **10** and **11** did not occur.

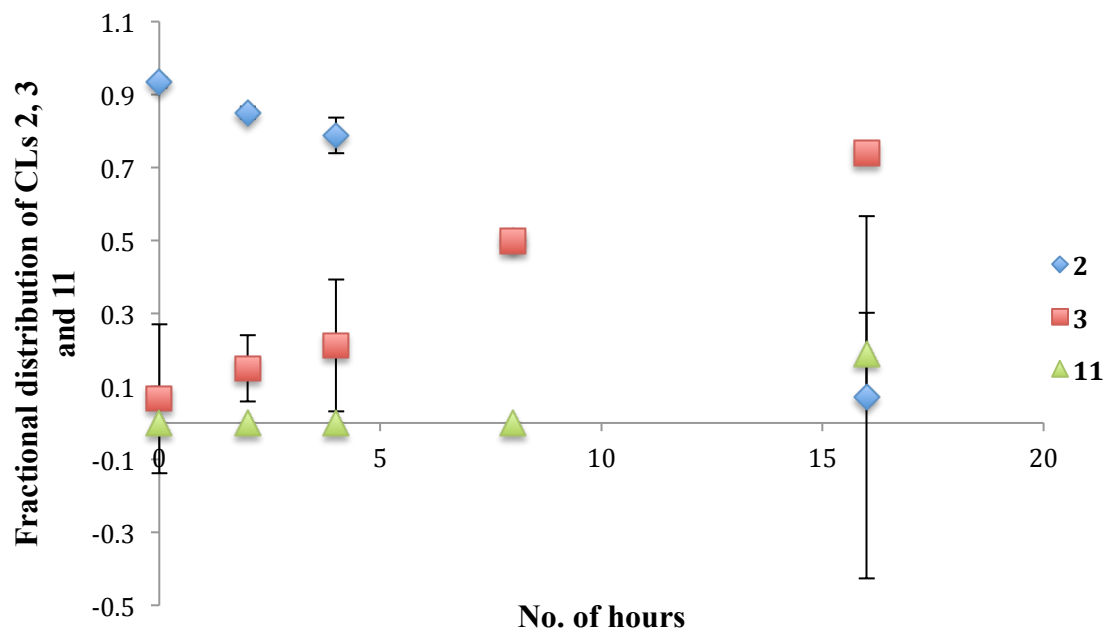


Figure 3.1 Extracted ion chromatogram analysis of CLs (**2**, **3** and **11**) showing the accumulation of **11** after 16 h

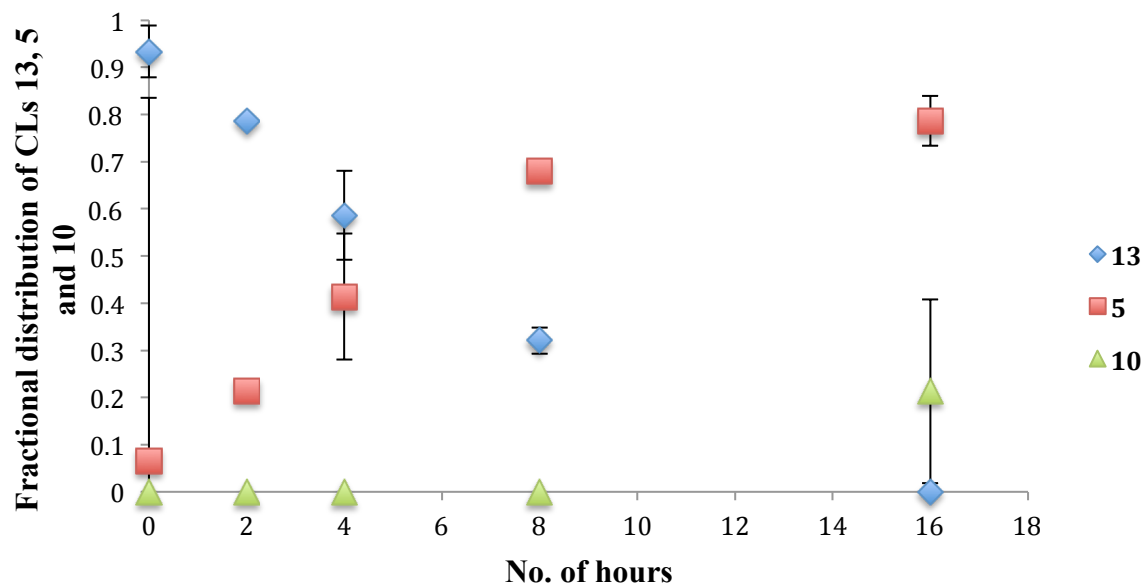


Figure 3.2 Extracted ion chromatogram analysis of CLs (**13**, **5** and **10**) showing the accumulation of **10** after 16 h

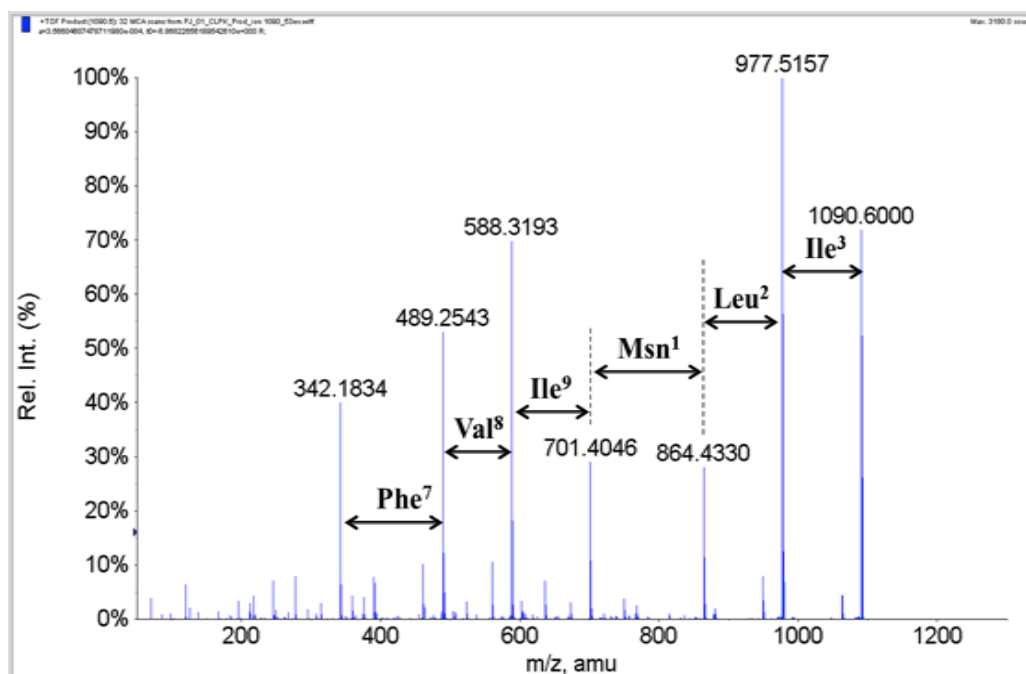


Figure 3.3 CID MS/MS spectra of **11** showing fragmentation pattern

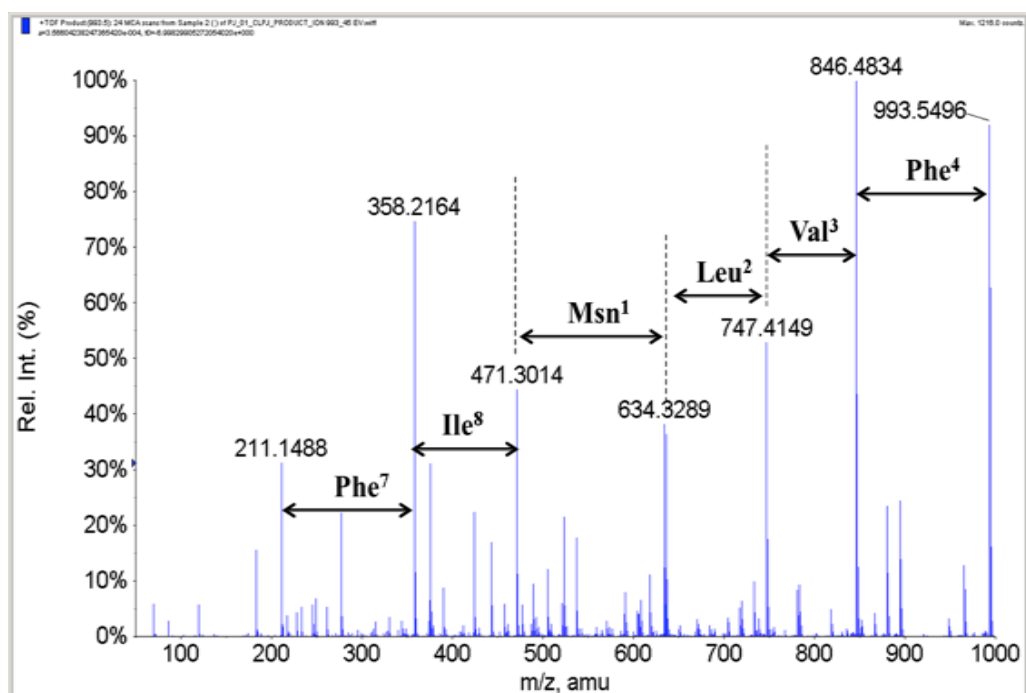


Figure 3.4 CID MS/MS spectra of **10** showing fragmentation pattern

3.4.3 Isolation of CLs from Flaxseed Oil

Oil-laden silica (Reaney, et al., 2013) (500 g) was mixed with hexane (1.5 L) and the slurry was filtered through a sintered glass funnel. The recovered silica was sequentially extracted with (a) 20% ethyl acetate in hexane (1.5 L \times 2), (b) ethyl acetate (1 L \times 3), and (c) 10% CH₃OH in CH₂Cl₂ (1 L \times 3). The filtrate from each fraction was concentrated *in vacuo* and subsequently subjected to HPLC-DAD analyses (214 nm). CLs **1**, **3**, **5**, **10** and **11** were detected in fractions b and c with retention times at 8.07, 12.12, 12.45, 16.05 and 21.27 min, respectively (Figure 3.5). Combined residues from fractions b and c were dissolved in methanol (250 mL), and then cooled to -20 °C for 3 h to allow a less soluble residue to settle. The methanolic solution was decanted and the residue was rinsed with methanol (150 mL \times 2) with cooling to -20 °C. The combined methanolic extracts were concentrated to produce a gummy residue that was sequentially extracted with hexane (150 mL \times 2) and diethyl ether (250 mL \times 2) at room temperature and at 0 °C, respectively. Finally, the residue was concentrated *in vacuo* to yield 37.5 g of crude CLs extract.

3.4.4 Synthesis of CLs 10 and 11

A stirred solution of the crude CLs extract (5.2 g) in CH₂Cl₂ (120 mL) was treated with *m*CPBA (3.75 g, 3 equivalents) at room temperature. After 1 h the reaction mixture was sequentially washed with 10% Na₂S₂O₃ (50 mL), saturated aq. NaHCO₃, and H₂O. The organic layer was concentrated to obtain a crude reaction mixture (4.2 g). The HPLC and HPLC-MS analyses indicated that the reaction mixture comprised CLs **1**, **10** and **11** with retention times at 12.32, 15.90 and 21.18 min, respectively (Figure 3.6). The crude reaction mixture was then subjected to preparative chromatography using BioCad Perfusion Chromatography Workstation to obtain CLs **1** (0.215 g), **10** (1.037 g), and **11** (0.997 g). All CLs were solvates. The solvent was removed by heating at 200 °C for overnight prior to elemental analysis. The experimental values of carbon, hydrogen, nitrogen and sulphur were: **1** (Anal calcd for C₅₇H₈₅N₉O₉: C- 65.27%, H- 8.28%, N- 11.89%; Found C- 65.81%, H- 8.24%, N- 12.12%); **10** (Anal calcd for C₅₁H₇₆N₈O₁₀S: C- 61.26%, H- 7.89%, N- 11.06%, S- 3.10%; Found C- 61.67%, H- 7.71%, N- 11.28%, S- 3.23%); **11** (Anal calcd for C₅₆H₈₃N₉O₁₁S: C- 61.32%, H- 7.75%, N- 11.37%, S- 2.80%; Found C- 61.69%, H- 7.67%, N- 11.56%, S- 2.94%).

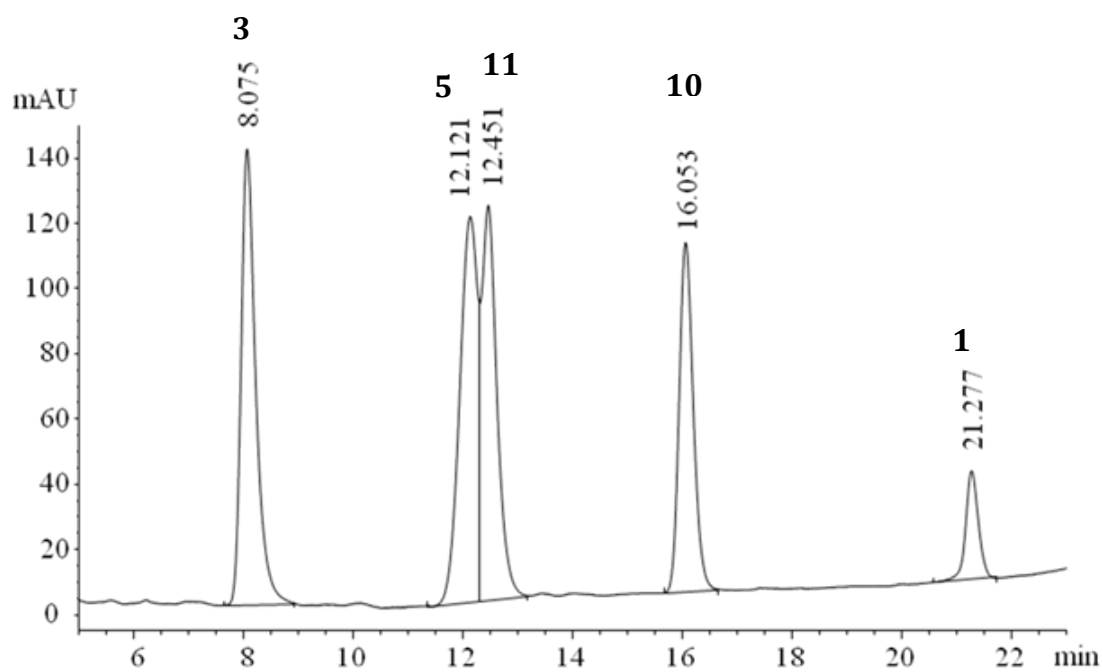


Figure 3.5 HPLC-DAD profile of CL mixture from silica gel extract

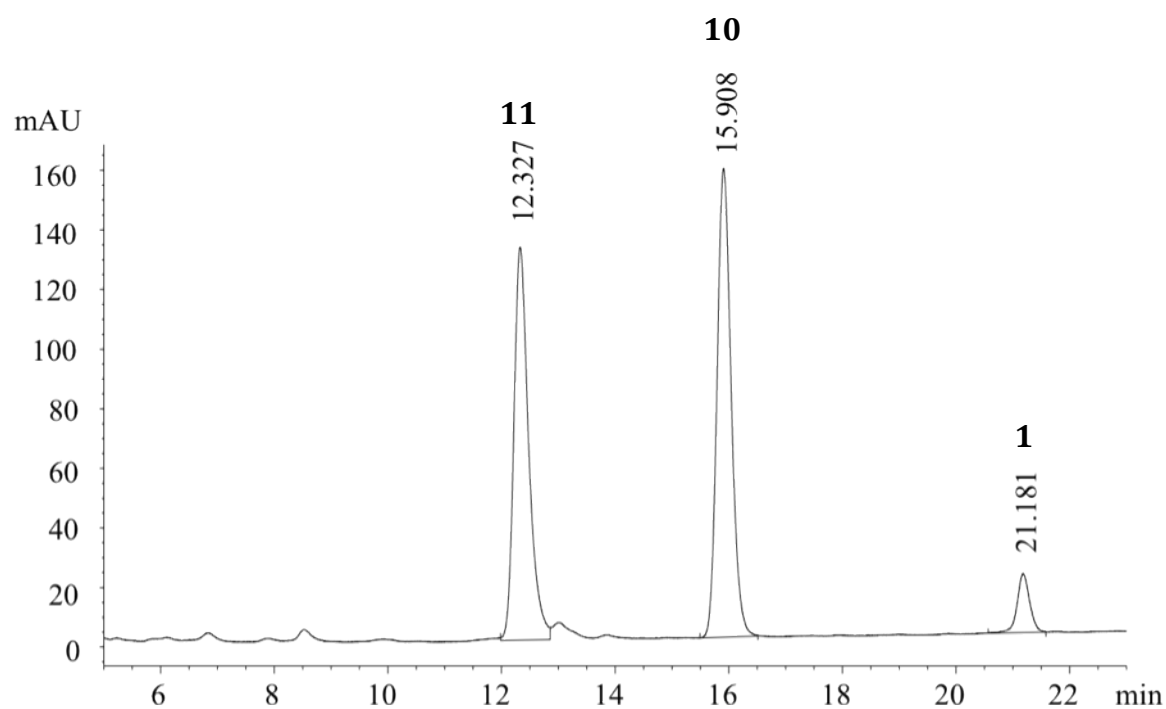


Figure 3.6 HPLC-DAD profile of CL mixtures following *m*-CPBA oxidation

3.4.5 Characterization of **11**

The molecular formula of **11** ($C_{56}H_{84}N_9O_{11}S$, quasimolecular ion signal at m/z 1090.5943 $[M+H]^+$) obtained by high-resolution HPLC-MS analysis was 15.9898 Da higher than that of **3** ($C_{56}H_{84}N_9O_{10}S$, m/z 1074.6045 $[M+H]^+$) thus suggesting presence of an additional oxygen atom. The IR absorption peaks at 3329 and 1667 cm^{-1} , characteristic of all known CLs, confirmed the presence of amide linkages in **11** whereas those at 1305 and 1133 cm^{-1} were attributed to the presence of a Msn residue in **11**. These absorption peaks are not diagnostic vibrations in the spectra of **2** or **3**. The structure analysis was further corroborated by tandem mass spectrometry (MS/MS) and NMR spectroscopic data. The MS/MS (Figure 3.3) and NMR spectral data of **11** showed remarkable similarities to that of **3** (Morita, et al., 1999). MS/MS fragmentation patterns revealed the presence of amino acid residues leucine (or isoleucine), valine, and two residues phenylalanine and proline. MS/MS doesn't distinguish between leucine and isoleucine. But 1H NMR data of **11** displayed most resonances present in **3**. ^{13}C NMR data showed nine amide carbonyl signals (δ 170.68, 171.27 (2), 175.46, 171.98, 173.08, 173.73 (2), 175.46) indicating a nonapeptide (Table 3.1). However, a fragment loss of m/z 163 in the MS/MS data of **11** (Figure 3.3) in place of the loss of m/z 147 (Mso in **3**) suggested the presence of an Msn residue in **11**. The sharp singlet at δ 2.85 ppm (signal at δ 2.45 ppm for **3** in DMSO) in the 1H NMR and carbon signal at δ 40.1 ppm (signal at δ 37.6 ppm for **3** in DMSO) in ^{13}C NMR data of **11** was thus attributed to the three protons and a carbon of a Msn moiety (Morita, et al., 1999). This singlet indicated that **11** existed as a single conformer under the NMR experimental conditions. The 1H NMR spectrum also showed six amide proton signals that were observed at δ 6.57, 6.80, 7.37, 7.57, 7.76, and 8.06 ppm (Table 3.1). The protons attached to the heteroatoms were assigned from 1H NMR, and their coupling to amide protons and carbonyl carbons employed NOE and HMBC correlations. These provided the amino acid sequence of the CL. Sequential assignment of β , γ and δ protons were done using 1H - 1H COSY and elucidation of the attachments of these protons to carbon atoms were performed by HMQC. The amino acid sequence of **11** was further established using tandem MS/MS profile (Figure 3.3) and was corroborated by 1H - 1H COSY. As shown in Figure 3.7, strong NOE correlations observed between $H\alpha$ protons of the two-prolyl groups confirmed cis geometry, defining rigidity within the molecule maintaining **11** in one stable conformation.

The structure of **11** was elucidated as *cyclo*-(Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile-). The optical rotation $[\alpha]_D^{22}$ of **11** was -85.7° (c 0.20, CH₃OH), compared to that of **3** at -109.7° (c 0.21, CH₃OH) that had similar amino acid sequence (Morita, et al., 1999).

3.4.6 Characterization of **10**

10 was subjected to similar spectroscopic characterization as **11**. **10** (C₅₁H₇₇N₈O₁₀S, m/z 993.5303 [M+H]⁺) also showed a mass 15.9899 Da higher than **5** (C₅₁H₇₇N₈O₉S, m/z 977.5404 [M+H]⁺) thus suggesting presence of an additional oxygen atom. The IR absorption maxima at 3329, 1667, 1305 and 1133 cm⁻¹ were similar to those of **11**. However, the ¹H NMR spectrum of **10**, unlike **11**, displayed two sharp singlets at δ 2.86 and 3.00 ppm that corresponded to Msn moieties in different environments. These observations suggested that **10** existed in two stable conformers under the NMR experimental conditions. However **5** showed only one Mso singlet at δ 2.56 ppm in DMSO (Morita, et al., 1999). The ¹H NMR revealed seven amide proton signals (δ 6.14, 6.86, 6.96, 6.98, 7.67, 7.71, and 7.72 ppm) (Table 3.2). The ¹³C NMR spectrum showed two sets of signals for each carbon atom corresponding to the two conformers, signals from the major conformer are shown in Table 3.2. Thus, eight amide carbonyl signals (δ 170.71, 171.06, 171.40, 171.80 (2), 172.72, 173.60, 174.90 ppm) were observed in ¹³C NMR, and MS/MS (Figure 3.4) fragmentation pattern suggested **10** to be an octapeptide. The NOE experiments (Figure 3.8) showed that the CL present as the major isomer is the trans prolyl isomer and the structure of **10** was confirmed to be *cyclo*-(Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile).

The optical rotation $[\alpha]_D^{22}$ of **10** was -79.3° (c 0.20, CH₃OH) compared to that of **5** at -75.5° (c 0.20, CH₃OH), both having similar amino acid sequences (Morita, et al., 1999).

3.4.7 Crystal Structure of **11**

We recently published the crystal structure of **11** as a butanol-water solvate that indicated eight trans amide bonds and one cis bond between two prolyl residues (Figure 3.9) (Jadhav, et al., 2011). It must be noted that butanol-water mixture provided hydrogen bond anchors for **11** that facilitated crystal formation. NOE shows strong coupling of H α of Pro₄ with H α of Pro₅ and coupling of N-H and H α of adjacent amino acids. Hence NOE results show cis bond between two proline residues and trans amide bonds among other residues. NMR data was collected in CDCl₃ at 320 K.

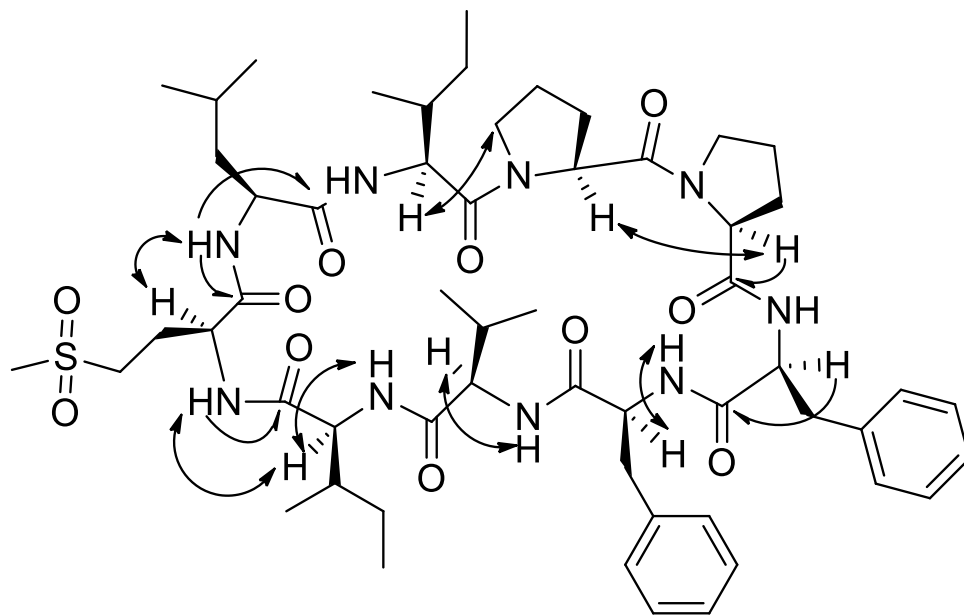


Figure 3.7 Structure of **11** (Double arrows show selected NOE correlations. Half arrows show selected HMBC correlations)

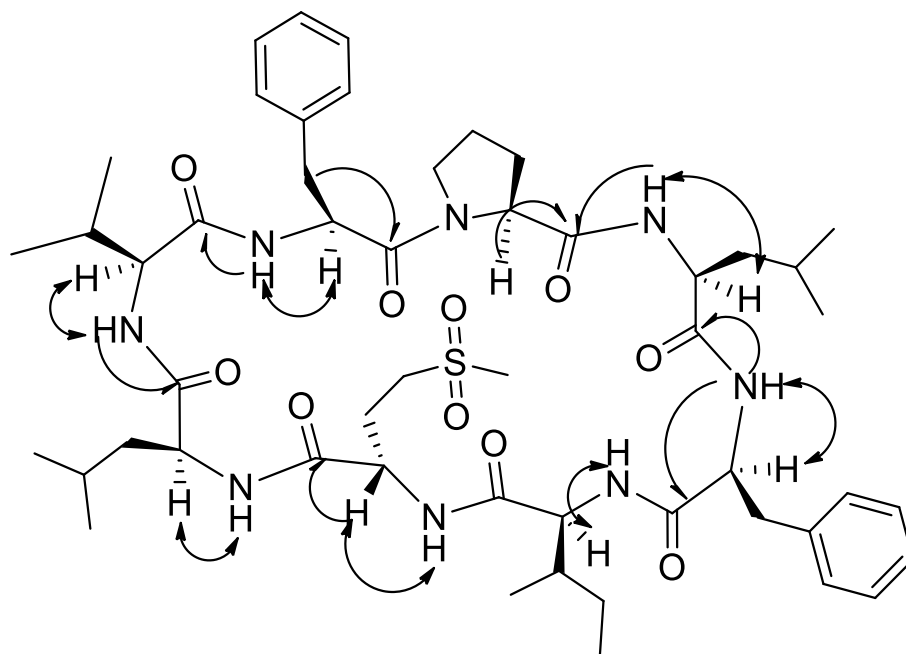


Figure 3.8 Structure of **10** (Double arrows show selected NOE correlations. Half arrows show selected HMBC correlations)

The 2D NMR investigative studies show that the solution structure of **11** is similar to that of its crystal structure. However, all attempts at crystallising **10** including the use of solvents such as butanol-water, methanol-hexane and butanol-hexane failed. This may be due to the existence of two conformers of **10** discovered in NMR experiments.

3.4.8 Thermal Stability of **10** and **11**

Additionally, we examined the acid and base stability of **10** and **11** at different temperatures in neat or in anhydrous organic solutions in a Kugelrohr apparatus. Some of the conditions were a) Neat at 200 °C for 2.5 h or 270 °C for 2 and 4 h; b) DMSO at 240 °C for 16 h; c) in tBuOK/THF at 100 °C for 4 h; and d) in trifluoroacetic acid at 200 °C for 4 h. Both CLs were stable under all the experimental conditions with no decomposition products detected. However, **5** and **3**, the parent CLs from which **10** and **11** are derived, decomposed to respective olefin isomers of 2-amino but-2-enoic acid and 2-amino but-3-enoic acid (data not shown). The result of these experiments showed that thermal stability of these CLs was significantly higher than their parent counterparts. Hence sulfone side chain and cyclic structural motif stabilizes these CLs at temperatures above 200 °C.

3.5 Conclusion

In summary, CLs **10** and **11** were detected in ground flaxseed during accelerated ageing. CLs (**1**, **3**, **5**, **10** and **11**) were isolated from flaxseed oil and derivatized to obtain gram quantities of **10** and **11**. The structures of **10** (trans prolyl isomer) and **11** were fully elucidated using high-resolution HPLC-MS-ESI, tandem MS, 1D (¹H and ¹³C) and 2D (NOE, COSY, and HMBC) NMR spectroscopic methods. This is the first report describing the complete characterization of **10** and **11**. It was established that **11** existed as a single conformer; perhaps by virtue of having two proline residues, whereas, **10** existed in two conformations. These pure compounds can be used as standards in food industry for detection of ageing of flaxseed and flaxseed products. Because of their chemical and oxidative stability **10** and **11** are good candidates in nanotubes and biomedical applications.

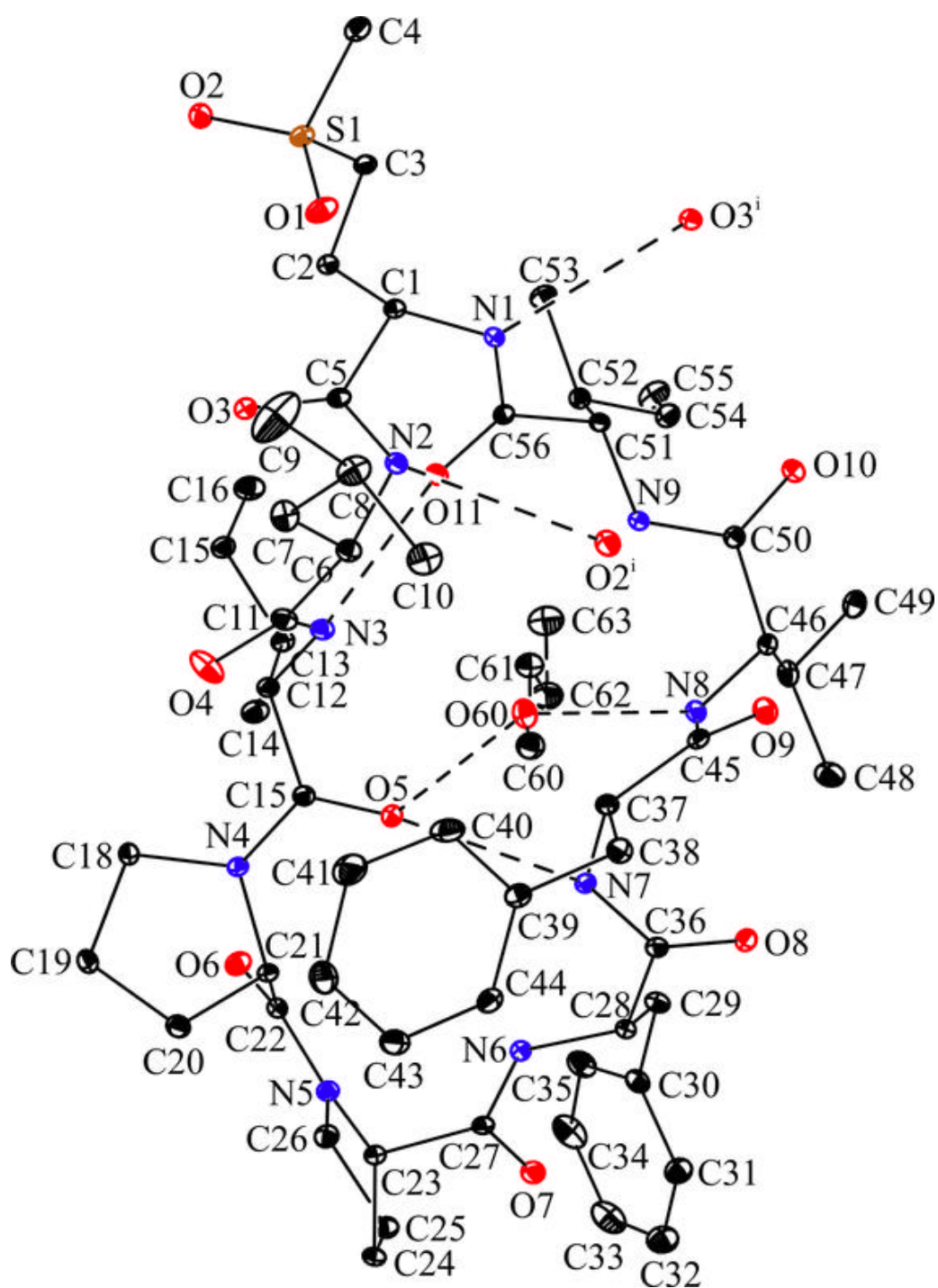


Figure 3.9 Crystal structure of **11** butanol-water solvate

3.6 Experimental Data

Table 3.1 ^1H and ^{13}C NMR assignments for **11**.

Assignment	δ_{H}	δ_{C}		δ_{H}	δ_{C}
Msn ¹					
α	4.81 (1H, m)	52.11	γ	1.50 (1H, m)	21.90
β	2.49 (1H, m)	26.13	δ	1.04 (1H, m)	
	2.32 (1H, m)			3.46 (1H, m)	47.35
γ	3.42 (1H, m)	51.09	C=O	3.31 (1H, m)	
	3.26 (1H, m)				173.73
ϵ_{Me}	2.85 (3H, s)	40.19	Phe ⁶		
NH	7.37 (1H, d, 8)		α	4.11 (1H, d, 8.4)	57.61
C=O		171.27	β	3.30 (1H, m)	36.72
			γ		135.95
Leu ²			δ	7.17 (2H, m)	129.21
α	4.07 (1H, m)	61.16	ϵ	7.27 (1H, m)	128.98
β	2.19 (1H, m)	38.39	ζ	7.33 (2H, m)	127.36
γ	2.05 (2H, m)	25.61	NH		
δ_{Me}	0.94 (6H, d, 6.6)	23.17	C=O		173.73
		22.05			
NH	7.76 (1H, d, 6.8)		Phe ⁷		
C=O		171.27	α	4.96 (1H, m)	53.83
			β	3.06 (1H, br.s)	35.59
Ile ³			γ		136.53
α	4.52 (1H, m)	56.49	δ	7.20 (2H, m)	129.88
β	1.96 (1H, m)	36.57	ϵ	7.27 (1H, m)	129.17
γ	1.52 (2H, m)	24.48	ζ	7.33 (2H, m)	127.50
γ_{Me}	1.09 (3H, d, 6.6)	16.02	NH	7.57 (1H, br.s)	
δ_{Me}	0.97 (3H, t, 6.6)	11.35	C=O		175.46
NH	8.06 (1H, br.s)				
C=O		171.37	Val ⁸		
			α	3.90 (1H, m)	59.07
Pro ⁴			β	2.14 (1H, m)	29.53
α	3.90 (1H, m)	62.02	γ_{Me}	0.86 (6H, m)	19.82
β	2.01 (1H, m)	28.63			18.43
	1.71 (1H, m)		NH	6.57 (1H, br.s)	
γ	2.05 (1H, m)	25.55	C=O		173.08
	1.95 (1H, m)				
δ	3.90 (1H, m)	48.11	Ile ⁹		
	3.67 (1H, m)		α	4.60 (1H, m)	58.54
C=O		170.68	β	1.99 (1H, m)	36.95
			γ	2.17 (2H, m)	25.27
Pro ⁵			γ_{Me}	0.95 (3H, d, 6.6)	16.24
α	4.07 (1H, m)	61.16	δ_{Me}	0.91 (3H, t, 7)	11.76
β	1.94 (1H, m)	32.21	NH	6.80 (1H, d, 8.4)	
	1.54 (1H, m)		C=O		171.98

Table 3.2 ^1H and ^{13}C NMR assignments for trans prolyl isomer of **10**.

Assignment	δ_{H}	δ_{C}		δ_{H}	δ_{C}
Msn¹			Pro⁵		
α	4.76 (1H, m)	51.65	α	4.09 (1H, m)	54.46
β	2.45 (1H, m)	25.65	β	1.72 (1H, m)	30.32
	2.20 (1H, m)			1.43 (1H, m)	
γ	3.28 (2H, m)	52.45	γ	1.67 (1H, m)	25.47
	3.00 (1H, m)			1.49 (1H, m)	
ϵ_{Me}	2.86 (3H, s)	40.68	δ	3.38 (2H, m)	46.72
$\epsilon_{\text{Me}}(\text{minor})$	3.00 (3H, s)	41.73	C=O		170.71
NH	7.72 (1H, br.s)				
C=O		171.06			
Leu²			Leu⁶		
α	4.36 (1H, m)	51.38	α	3.81 (1H, m)	55.39
β	2.33 (1H, m)	25.56	β	2.20 (1H, m)	37.19
	1.79 (1H, m)			1.86 (1H, m)	
γ	1.95 (1H, m)	32.38	γ	1.56 (1H, m)	25.38
δ_{Me}	0.90 (3H, d, 6.2)	23.20	δ_{Me}	0.96 (3H, m)	22.84
	0.84 (3H, d, 6.2)	22.29		0.90 (3H, m)	22.13
NH	6.96 (1H, d, 9)		NH	7.67 (1H, d, 7)	
C=O		171.80	C=O		173.60
Val³			Phe⁷		
α	4.50 (1H, dd, 6.7, 6.7)	57.70	α	4.32 (1H, m)	58.79
β	2.05 (1H, m)	31.16	β	3.14 (1H, m)	37.49
γ_{Me}	0.96 (3H, d, 6.6)	19.57		3.06 (1H, m)	
	0.90 (3H, d, 6.6)	18.50	γ		135.66
NH	6.98 (1H, d)		δ	7.24 (2H, m)	129.40
C=O		171.80	ϵ	7.27 (1H, m)	129.33
Phe⁴			ζ	7.38 (2H, m)	128.83
α	4.34 (1H, m)	53.37	NH	7.71 (1H, br.s)	
β	3.03 (2H, m)	39.02	C=O		174.90
γ		135.76	Ile⁸		
δ	7.20 (2H, m)	129.85	α	4.13 (1H, m)	60.41
ϵ	7.27 (1H, m)	129.69	β	1.88 (1H, m)	35.93
ζ	7.33 (2H, m)	128.88	γ	1.57 (2H, m)	25.59
NH	6.86 (1H, d, 8.4)		γ_{Me}	0.84 (3H, d, 6.2)	16.51
C=O		171.40	δ_{Me}	0.99 (3H, t, 6.2)	11.87
			NH	6.14 (1H, br.s)	
			C=O		172.72

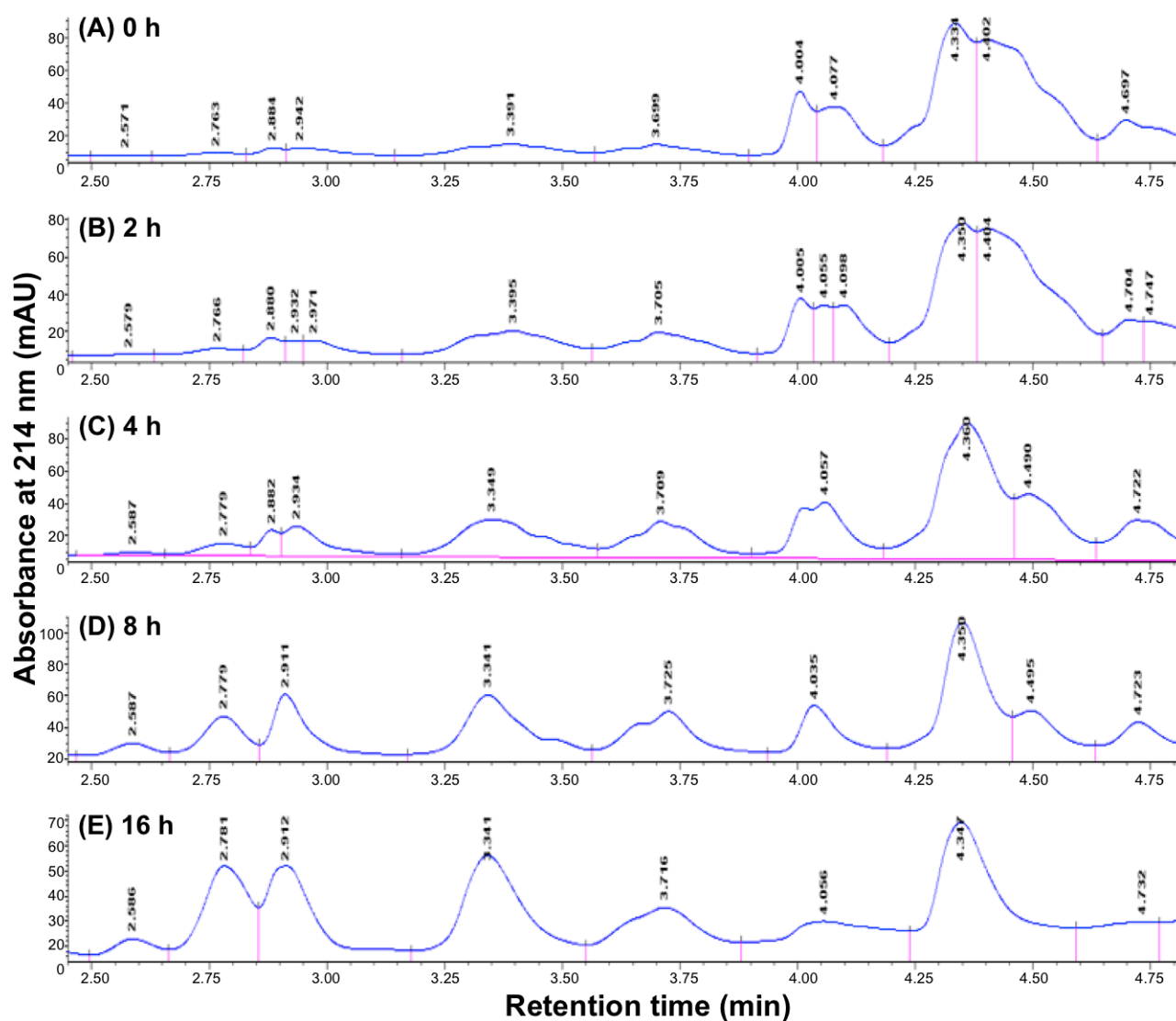


Figure 3.10 HPLC chromatograms of CLs during accelerated ageing of flax

Table 3.3 Data extracted from HPLC chromatograms of CLs during accelerated aging flax.

Time	Retention times (CL)-
0 h	2.94 (3), 3.39 (5), 3.69 (4), 4.00 (2), 4.33 (14,1,13), 4.40 (15), 4.69 (12)
2 h	2.76 (7), 2.93 (3), 3.34 (5), 3.71 (4), 4.00 (2), 4.35 (14,1,13), 4.40 (15), 4.70 (12)
4 h	2.77 (7), 2.93 (3), 3.34 (5), 3.70 (4), 4.05 (2), 4.36 (14,1,13), 4.49 (15), 4.72 (12)
8 h	2.58 (6), 2.77 (7), 2.91 (3), 3.34 (5), 3.72 (4), 4.03 (2), 4.36 (14,1,13), 4.49 (15), 4.72 (12)
16 h	2.58 (6), 2.78 (7), 2.91 (3), 3.34 (5,11), 3.71 (4,10), 4.05 (2), 4.34 (1,13)

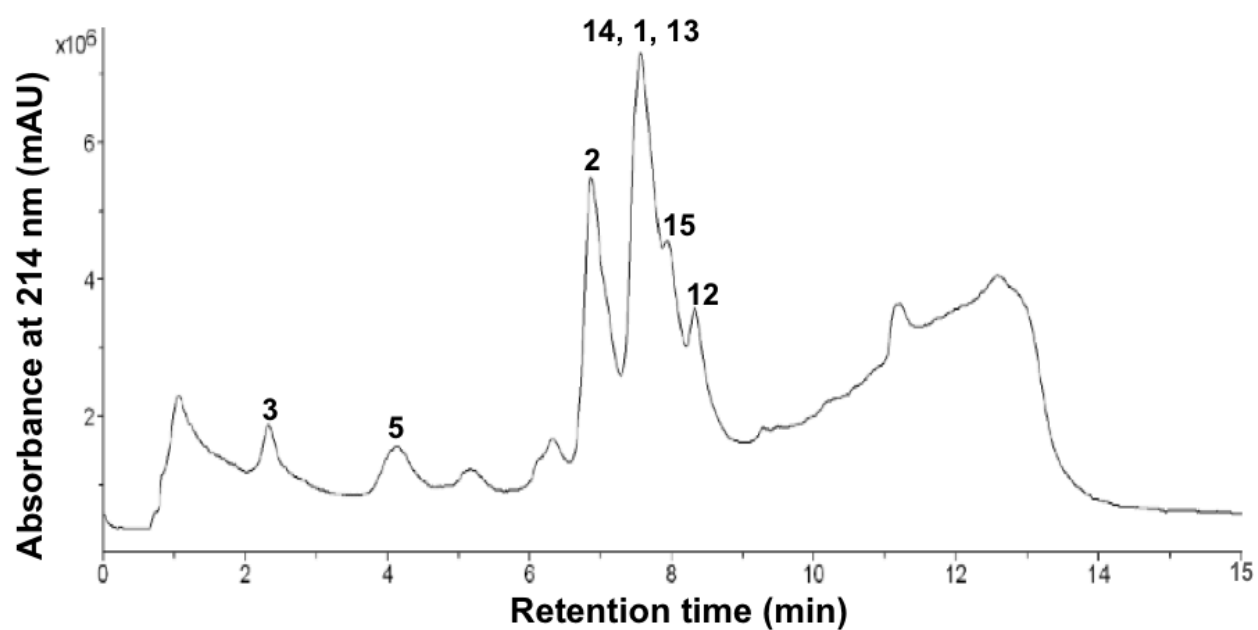


Figure 3.11 LC-DAD trace of CLs in freshly ground flax meal

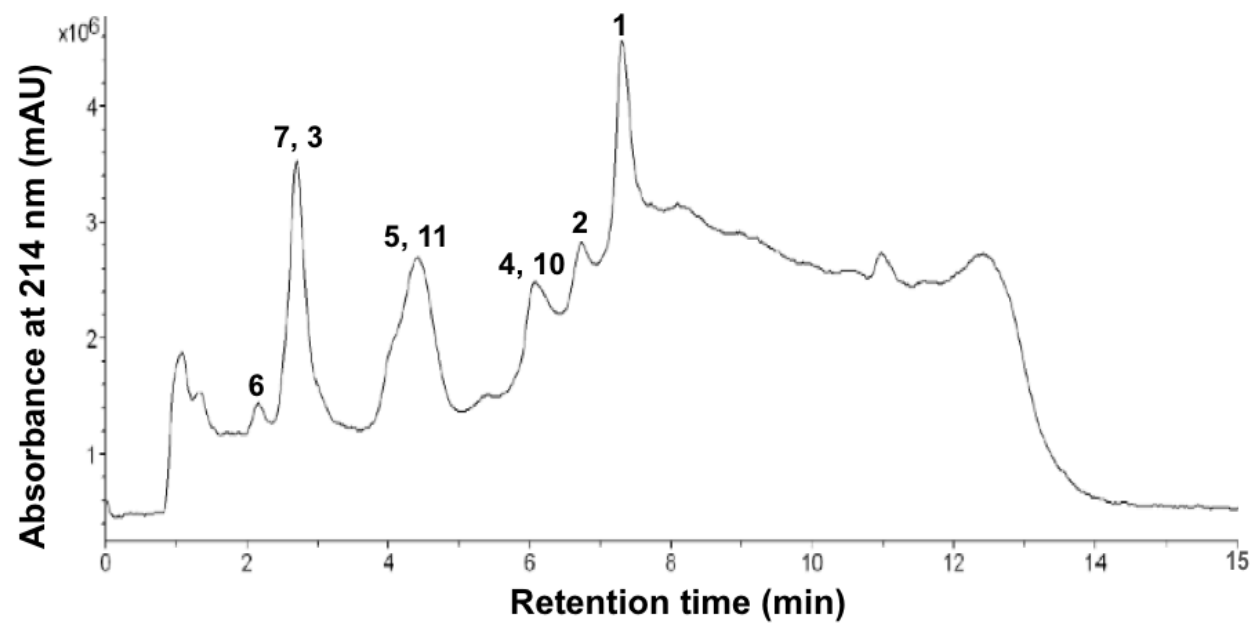


Figure 3.12 LC-DAD trace of CLs in ground flax meal during accelerated ageing after 16 h

3.7 Brief Introduction to Chapter 4

As described in Chapter 3, individual CLs (**1**, **10** and **11**) are extracted, isolated and purified in multigram quantities from flax oil. CLs (**10** and **11**) containing Msn were also detected in ageing flaxseed. The main aim of this chapter is to develop synthetic protocols to chemically modify CLs from flax. It is likely that modification of the CLs will be necessary in order to increase their utility before they can be effectively used in any application. It was found that CLs **10** and **11** are stable to acid and base at temperatures above 100 °C (Chapter 3). Hence the chemical modification of **10** and **11** is tedious. They are oxidized products of CLs (**5** and **3**) containing Mso and CLs (**13** and **2**) containing Met. A new branch of peptide modification through sulfur chemistry has been developed. Met side chain of CLs (**13** and **2**) readily reacts with organo halides to form novel substituted Met analogs. It was found that Met and Mso derivatives of these analogs were not stable at higher temperatures. In addition, Mso derivatives are chiral in nature and may increase the complexity of the reaction product mixture. Hence the modified Met was oxidized to more stable Msn derivative to take advantage of the thermal stability and simple structure of Msn. Finally Msn derivative was modified to obtain analogs containing modified Msn side chains with –NH₂ and –OH groups in a systematic fashion by keeping basic core intact. In addition, methods were developed for the separation of CLs from reaction mixtures after modification. These novel derivatives were characterized using NMR and MS. The delta shifts of α protons of the amino acids were used to compare the peptide backbone structures of parent CLs and their derivatives. Analogs of **11** have shown slight shift perturbation as **11**, while analogs of **10** shows major shift perturbation. These chemically modified CLs will allow utilizing them in different applications.

CHAPTER 4

SITE-SPECIFIC MODIFICATION OF CYCLOLINOPEPTIDES THROUGH METHIONINE

4.1 Abstract

Flax CLs **10** and **11** are reported to have immunosuppressant activity. While it is desirable to produce analogs of these CLs and link them to other molecules they lack active nuclei that are commonly used in peptide modification. The methyl sulfide present in methionine of the parent CL may be converted to a sulfonium, which can be used in producing more reactive derivatives. We found that this route preserves the integrity of the CL. Analogs of **2**, **3**, **5**, **10**, **11** and **13** with activated groups such as -CN, -COOEt, and -NH₂ were synthesized from **2** and **13**. These compounds were characterized by 1D and 2D NMR spectroscopy. It was observed that methionine (SCH₃) and methionine sulfoxide (SOCH₃) were easily eliminated with heating. These compounds were not suitable for production of stable analogs. Hence they are oxidized to more stable methionine sulfone analogs. Methionine modified CLs that contain active groups can be used for synthesis of CL adduct complexes. Such adducts include fluorescent tagged CLs, affinity chromatography media, and haptens suitable for antibody production.

4.2 Introduction

Cyclic peptides belong to important class of naturally occurring biologically active compounds. For example, gramicidin S (Kratzschmar, et al., 1989) and tyrocidine A (Mootz & Marahiel, 1997) are used as antibiotics whereas cyclosporine A (Emmel, et al., 1989) is used as an immunosuppressant drug. Cyclic peptides are more stable than linear peptides and resist protease degradation, For example, cyclic Gai binding peptide [*cyclo*-(MITWYEFVAGTK)] have shown proteolytic stability compared to the parent linear molecule. (Driggers, et al., 2008; Kleiner, et al., 2010; Millward, et al., 2007). Selective transformation of functional groups on biomolecules such as carbohydrates and peptides is important in biomedical research to prepare adducts and study structure-function relationships. In addition, tagged peptides were also used in forming fluorescent adducts, affinity media, isotope labeled peptides and haptens for antibody production (Bode, et al., 2012; Hamamoto, et al., 2011; Jayaraj, et al., 2003; Tozzi, et al., 2003). However, site directed chemical modification without affecting other functional groups is often a formidable challenge. Modified cyclic peptides may be produced by several approaches. In a classical approach, amino acids are sequentially added to a solid phase support, and then released by a reaction that results in cyclization (Merrifield resin) (Merrifield, 1963, 1965, 1969). A second approach requires a source of peptide that can be modified through a heteroatom such as oxygen, sulfur or nitrogen found in suitable amino acids including serine, threonine, cysteine, lysine etc. (Kotha & Lahiri, 2005). The latter approach is particularly efficient in peptides that have few heteroatoms and, thus, do not need the addition of protective groups to convert carboxylic and amine and active side group functionality of the peptides to less reactive species. A further advantage of this strategy is realized with mild reactions that preserve the chirality of the peptides. This approach is suited for studies of structure/function activity relationships of biologically active peptides to generate families of analogs (Jamonnak, et al., 2010). There are 13 proteinogenic amino acids containing hetero functional groups in their side chains, which may undergo chemical transformations under mild conditions. For examples, alkylation of thiol functionality in a cysteine residue of an unprotected tetrapeptide has been reported (Or, et al., 1991) also the hydroxyl groups of serine have been used as reactive centers in elimination-addition (Sommerfeld & Seebach, 1993) and metal-crosscoupling reactions (Dunn, et al., 1995).

The alkylation of methionine (Met) residues in chymotrypsin and ribonucleaseA were previously reported using α -halo acetic acids and derivatives (Gundlach, et al., 1959a; Lawson & Schramm, 1962). Iodoacetic acid can alkylate sulfhydryl groups at neutral or slightly acidic pH at faster rates compare to other nucleophilic amino acid residues like lysine, tyrosine and histidine (Korman & Clarke, 1956). The reaction of Met and iodoacetic acid yields a sulfonium salt, and elemental analysis and decomposition products formed during acidic hydrolysis confirmed the formation of salt. Depending on the reaction conditions, different hydrolysate products were formed from sulfonium salt. Alkylation of L-methionine at 110 °C in 6 N HCl for 20 h leads to S-carboxymethylhomocysteine. In addition, the sulphonium salt decomposes to homoserine at 100 °C for 1 hour in the presence of 0.2 M phosphate buffer (pH 6.5). Similar conditions in 0.2 M citrate buffer at pH 2.2, homoserine lactone was formed along with homoserine. The decomposition of Met based sulfonium salts depends on bonding of the Met through its carboxyl and amino groups. For example, the alkylation of L-methionine is pH independent and Met desulfurizes to homoserine and homoserine lactone during acid hydrolysis. However, Met residue of ribonuclease A efficiently alkylates at pH 2.8 and 40 °C. After hydrolysis, Met, S-carboxymethylhomocysteine and homoserine (and its lactone) products were formed (Gundlach, et al., 1959a; Gundlach, et al., 1959b). It was also reported that Met can be regenerated from sulfonium salts using β -mercaptoethanol (Naider & Bohak, 1972). This approach was used for isolation of Met peptides under acidic conditions (Grunert, et al., 2003).

Flaxseed oil contains hydrophobic CLs comprising eight or nine amino acids. CLs are bioactive compounds with potential for use as therapeutics. To date, nine CLs (**1** to **9**), having molecular weights of approximately 1 kDa have been isolated from flaxseed (Figure 2.6 and Table 2.3, Chapter 2) (Kaufmann & Tobschirbel, 1959; Matsumoto, et al., 2001; Morita, et al., 1999). Reduced CLs **12–15** of CLs **4–7** respectively have also been detected in flax oil and characterized using tandem MS fragmentation patterns (Stefanowicz, 2001). The CLs **1**, **3**, and **5** induce apoptosis in a human lung epithelial cancer cell line (Reaney, et al., 2013). **1** inhibits both cholate uptake into hepatocytes of rat liver (50% inhibition at 3 μ m) and the activation and proliferation of T-lymphocyte. Other compounds, which are similar in structure, such as somatostatin and antanamide have also shown inhibition of cholate uptake into hepatocytes. (Gaymes, et al., 1996; Kessler, et al., 1986). The two newly discovered CLs **10** and **11** possess immunosuppressive activity (Figure 2.6 and Table 2.3, Chapter 2). Of all the eleven CLs, **1**

showed the best immunosuppressive activity on concanavalin A induced response of human peripheral blood lymphocytes with an IC_{50} of 2.5 $\mu\text{g/mL}$ followed by **11** (25.2 $\mu\text{g/mL}$) and **10** (28.1 $\mu\text{g/mL}$) (Morita & Takeya, 2010). The CLs **10** and **11** were synthesized in 75% isolated yield *via* oxone oxidation of **5** and **3**, respectively (Matsumoto, et al., 2001), but were also detected in heat treated flax meal at 100 °C with constant air pressure of 60 kPa in the heating block of an OSI instrument for 16 h (Jadhav, et al., 2013).

Analogues of **1** were produced using an amino acid synthesizer and substituting amino acids in the structure (Siemion, et al., 1999). Zabrocki et al. synthesized various analogues of **1** using β^3 -Phe, homoPhe, β -Pro amino acid residues and conducted structure activity relationship studies of the immunosuppressive activity. Mouse splenocytes and sheep erythrocytes were used to measure proliferative response and secondary humoral immune response *in vitro* (Drygała, et al., 2009; Kaczmarek, et al., 2009; Katarzyńska, et al., 2008). However, **1** has shown better immunosuppressive activity as compared to its various analogues. It was suggested that the biological activity of **1** might reside in the Pro-Phe-Phe tripeptide fragments in the cyclic chain. It was also found that the amino acid triad Pro- X_{xx1} - X_{xx2} was also present in other immunosuppressant cyclic peptides such as cycloamanide B (Pro-Ser-Phe), antamanide (Pro-Phe-Phe) and hymenistatin I (Pro-Tyr-Val) where X_{xx1} can be a hydrophobic and X_{xx2} can be a hydrophobic or an aromatic amino acid residue (Kessler, et al., 1986; Siemion, et al., 1999). A similar tripeptide fragment also occurs in CLs **10** and **11** where X_{xx1} is Phe in **11**, but Leu in **10**. Hence during the chemical modification of **10** and **11**, this bioactive tripeptide fragment did not retain its activity when modified.

This chapter describes the site-specific modification of CLs **3** and **5** through the Met residue while maintaining the CLs core structure intact. Modification of cyclic peptides through Met has not been previously reported and hence various analogues were synthesized and characterized. In addition, these modified CLs were completely characterized using spectrometric techniques including 1D and 2D NMR.

4.3 Materials and Methods

All chemicals were purchased from Sigma-Aldrich and HPLC grade solvents were used. **3** and **5** were obtained from flax oil (Okinyo-Owiti, et al., 2013).

All other reagents were purchased from commercial sources and were used without further purification. The NMR spectra were recorded on a 500 MHz Bruker Avance spectrometer (Bruker Biospin, Germany, Inverse triple resonance probe, TXI, 5 mm, XWIN-NMR 3.5 software). Well resolved sharp spectral signals were obtained from NMR spectra acquired at 320 K. Subsequently, the 2D NMR spectra were also acquired at 320 K. Proton and corresponding carbon signals were assigned using ^1H - ^1H COSY and HMQC correlations. The correlations between the amide protons, carbonyl carbons and α -CH protons were determined from NOE and HMBC experiments. ^1H NMR spectra were taken in CDCl_3 and referenced to the appropriate solvent signal. Chemical shifts are reported in ppm units downfield from tetramethylsilane. Fourier transform infrared (FT-IR) spectra were recorded on a Bio-Rad FTS-40 spectrometer (Win-IR software, Version-4.14) using the diffuse reflectance method on samples dispersed in KBr (DRIFT); only diagnostic and/or intense peaks are reported.

HPLC-DAD analysis was performed with an Agilent 1200 series HPLC system equipped with a quaternary pump, autosampler, degasser and diode array detector (Agilent G1315C/D, 1024-element photodiode array, wavelength range 190–300 nm). The peaks were detected at wavelengths of 214 nm with a 10 nm bandwidth and against a reference signal at 300 nm. Chromatography was conducted using a Chromolith[®] SpeedRod RP-18e column (Sorbent lot/column No.-UM8142/034, 2 μm mesoporous size, 50 \times 4.6 mm, i.d.) equipped with an online filter. The mobile phase consisted of H_2O -acetonitrile (70:30 to 30:70 in 4 min, 10:90 in 0.5 min, 70:30 in 0.5 min and equilibrated to 70:30 for 1 min) at a flow rate of 2 mL/min (Olivia, et al., 2012). A Chromolith[®] SemiPrep RP-18e column (Sorbent lot/column No.-UM8707/016, 2 μm particle size, 100 \times 10 mm i.d.) equipped with an online filter was used for preparative liquid chromatographic separations of the CLs. The mobile phase consisted of H_2O -acetonitrile (80:20 for 0.5 min and to 10:90 in 4.5 min, 80:20 in 0.25 min and equilibrated to 80:20 for 0.75 min) at a flow rate of 5 mL/min.

Reverse phase chromatography of crude CLs reaction mixtures were also performed using Sep-Pak[®] vac 20 cc (5 g) tc_{18} cartridges (37–55 μm particle size, 125 Å, Waters, Ireland). The mobile phase consisted of gradient elution of H_2O -acetonitrile (100:0 to 0:100).

Mass spectral analyses were achieved using a MicroTOF-Q II Mass Spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an atmospheric-pressure chemical-

ionization (APCI) source (Parameters- dry temp- 200 °C; vaporizer temp- 450 °C; dry gas- 8 L/min; nebulizer 1.6 bar; capillary voltage- 4000 V) and used for MS/MS of modified CLs. Further high resolution HPLC-MS was performed on an Agilent HPLC 1100 series directly connected to QSTAR XL Systems (Mass Spectrometer Hybrid Quadrupole-TOF LC-MS/MS, Applied Biosystems, Toronto, Canada) with electrospray ionization (ESI) source. Chromatographic separation was achieved at room temperature using a Hypersil ODS C-18 (Hewlett-Packard, Germany, 5 µm particle size silica, 100 × 2.1 mm) column. The mobile phase consisted of a linear gradient of solvent A, H₂O containing 0.1% formic acid, and solvent B, acetonitrile containing 0.1% formic acid. The elution was performed using a linear gradient from 55% of B to 90% of B within 25 min at a flow rate of 0.25 mL/min.

4.4 Results and Discussion

In the current investigation, CLs **10** [*cyclo*-(Msn-Leu-Val-Phe-Pro-Leu-Phe-Ile)] and **11** [*cyclo*-(Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)], each containing a methyl sulphone (Msn) residues are biologically active (Morita & Takeya, 2010) and thermally stable (Jadhav, et al., 2013). But the attempts to reduce Msn to Met were unsuccessful (data not shown). The Met containing CLs **2** and **13** were, therefore, accessed through reduction of the methionine sulfoxide (Mso) precursor CLs **3** and **5**, respectively. Mso containing peptides are readily reduced to Met containing peptides using (NH₄I/TFA/Me₂S) and (TiCl₄/NaI) (Nicolas, et al., 1995; Pennington & Byrnes, 1995) and, thereby, allowing modification of these compounds with reactive iodomethylene groups (Gundlach, et al., 1959a). The Met therefore serves as point of attachment for the elongation of activated side chain while keeping the basic core of CL structure intact. CLs containing reactive groups such as groups–NH₂, –COOH and –OH may thus be readily incorporated thus enabling selective coupling with other reactive functional groups.

4.4.1 Optimization of Met Coupling

CL **3** was reduced to **2** using sodium borohydride in presence of iodine (Figure 4.1). Met is reactive and can be reacted with electrophilic haloalkyl or haloacetyl compounds to form additional compounds (Kramer & Deming, 2012). The plausible mechanism for the alkylation of Met with electrophilic group (R-X) is through the formation of a sulfonium intermediate and subsequently release of the MeX leaving group forming a S-R compound (Gundlach, et al., 1959a). CL **2** was subsequently treated with iodoacetonitrile in presence of dimethylformamide

(DMF) and *N*-methyl pyrrolidinone at 100°C for 20 h to form nitrile **16** (-SCH₂CN). Similar treatment with iodoacetate yielded ester **21** (-SCH₂COOEt). Met compounds can be eliminated using Raney Ni to alkane (α -amino butyric acid) (Kiryushkin, et al., 1969) or to produce an alkene (S-vinyl glycine) at elevated temperature (Kuechenthal, et al., 2010).

In our attempts to reduce nitrile **16** to an amine led to elimination of mercaptoacetonitrile to form **17** under the experimental conditions. In order to avoid elimination product, **16** was oxidized to sulfoxide nitrile (**18**) (-SOCH₂CN). However, the sulfoxide nitrile **18** underwent elimination under reducing conditions (H₂ at 1 atm, Pd-C and LiOH at 170 °C) to produce mixtures of external and internal olefins. It was surmised that oxidation of **16** to a more stable sulfone **19** (-SO₂CH₂CN) will provide access to amine **20** that resists elimination. Thus, *m*CPBA oxidation of **16** yielded **19** that was reduced to amine **20** (-SO₂CH₂CH₂NH₂) under 1 atm of H₂, PtO₂ and AcOH at room temperature in low yield. Elimination products were not detected under these reaction conditions.

An alternative and better yielding route to amine **23** and alcohol **24** that does not lead to elimination products was sought. **23** and **24** were obtained from ester **21** following its oxidation to a comparatively thermally stable **22**. Treatment of **22** with ethylene diamine and ethanolamine provided access to amine **23** and alcohol **24** respectively. Similarly the amine **27** and alcohol **28** analog of **10** were also synthesized (Figure 4.2).

4.4.2 Characterizations of **24** and **28**

The structures of **24** and **28** were confirmed by MS/MS and NMR methods (Figure 4.3). HPLC-MS analysis showed a [M+H]⁺ ion peak at *m/z* 1177.6730 corresponding to a molecular formula, C₅₉H₈₉N₁₀O₁₃S of **24** and *m/z* 1080.5921 [M+H]⁺ corresponding to a molecular formula, C₅₄H₈₂N₉O₁₂S of **28**. MS/MS fragmentation pattern shows the amino acid sequence of both these derivatives (Figure 4.4) and also confirms the attachment of hydroxyl side chain to the Msn residue. The IR absorption peaks at 3329 cm⁻¹ and 1665 cm⁻¹, characteristic of all known CLs (Jadhav, et al., 2013; Matsumoto, et al., 2001; Morita, et al., 1999) confirmed the presence of amide linkages in **24** and **28** whereas those at 1320 and 1122 cm⁻¹ were attributed to the presence of a Msn residue and broad band at 3334 shows presence of hydroxyl groups in **24** and **28**. The CID MS/MS and NMR spectral data of **24** (Figure 4.4A) showed remarkable similarities to that of **11**.

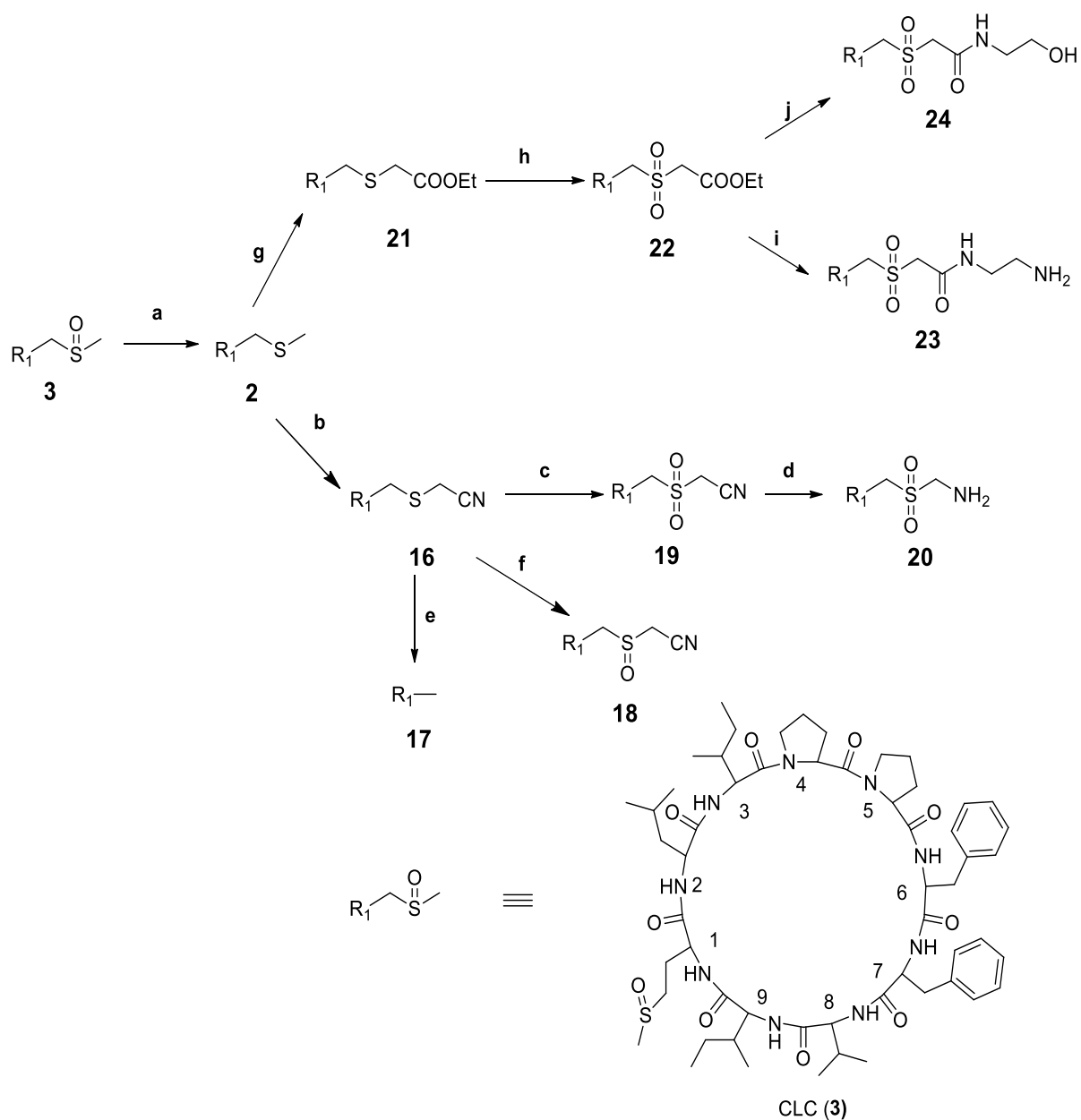


Figure 4.1 Synthesis of amine and alcohol derivatives of **11**. Reagents and conditions: a) $NaBH_4$, I_2 , THF, room temperature (RT); b) ICH_2CN , DIPEA, NMP, 100 °C; c) *m*CPBA, DCM, RT; d) H_2 (1 atm), PtO_2 , AcOH, RT; e) H_2 (1 atm), Pd-C, LiOH, dioxane: water, 140°C; f) $NaIO_4$, THF: H_2O ; g) ICH_2COOEt , DIPEA, NMP, 100 °C; h) *m*CPBA, DCM, RT; i) ethylenediamine, MeOH, reflux; j) ethanolamine, MeOH, reflux

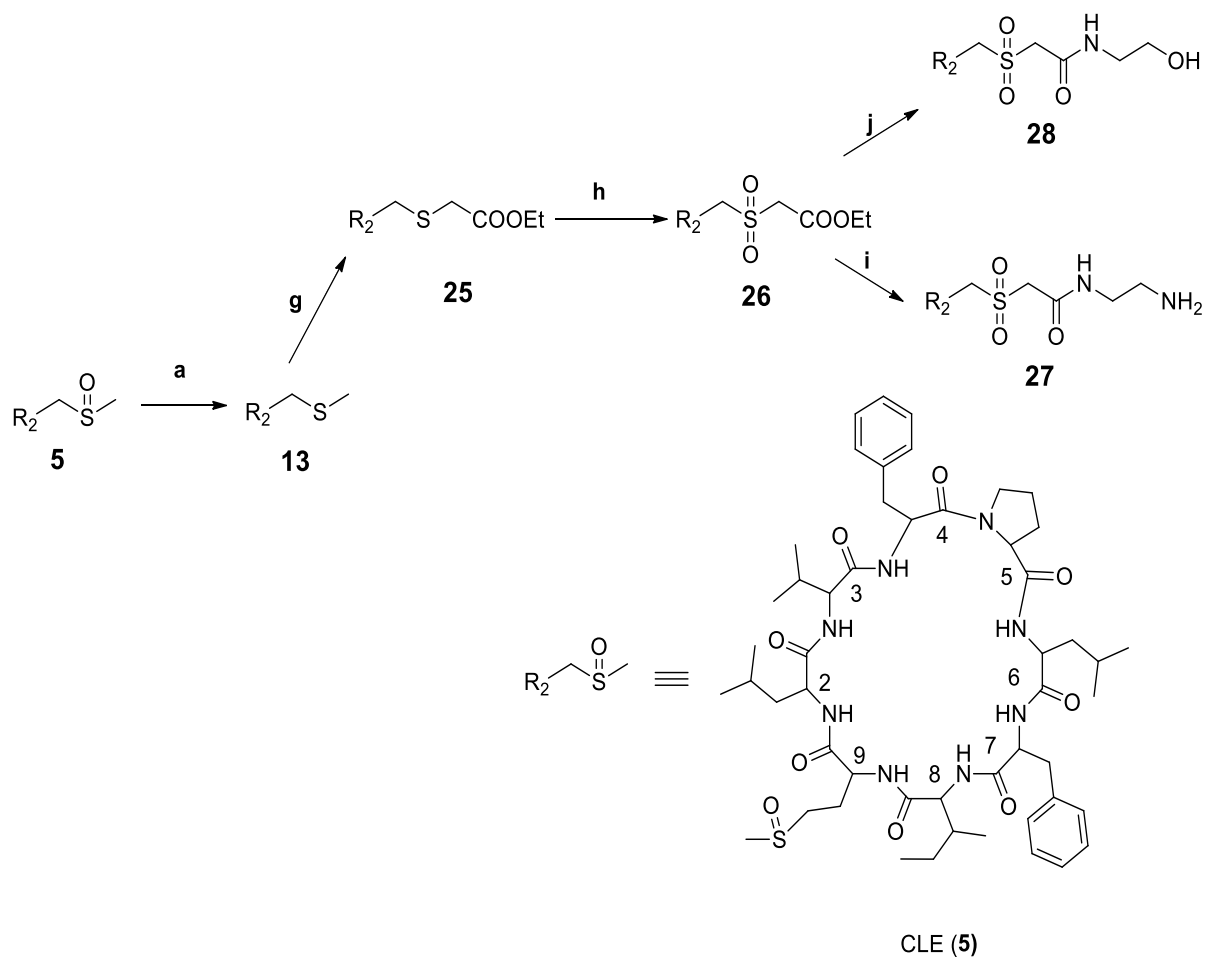


Figure 4.2 Synthesis of amine and alcohol derivatives of **10**. Reagents and conditions: a) NaBH_4 , I_2 , THF, RT; b) ICH_2CN , DIPEA, NMP, $100\text{ }^\circ\text{C}$; c) *m*CPBA, DCM, RT; d) H_2 (1 atm), PtO_2 , AcOH, RT; e) H_2 (1 atm), Pd-C, LiOH, dioxane: water, $140\text{ }^\circ\text{C}$; f) NaIO_4 , THF: H_2O ; g) ICH_2COOEt , DIPEA, NMP, $100\text{ }^\circ\text{C}$; h) *m*CPBA, DCM, RT; i) ethylenediamine, MeOH, reflux; j) ethanolamine, MeOH, reflux

However, a fragment loss of m/z 250 in the MS/MS data of **24** (Figure 4.4A) in place of the loss of m/z 163 (Msn in **11**) suggested the presence of an alcohol bearing side chain attached to the Msn residue in **24**. The α protons were assigned from ^1H NMR while β , γ and δ protons were assigned using ^1H - ^1H COSY. Elucidation of the attachments of these protons to carbon atoms as performed by HMQC. In addition, the coupling of amide protons with carbonyl carbons was confirmed using NOE and HMBC correlations (Figure 4.3). These data provided the amino acid sequence of the CL. The amino acid sequence of **24** was further established using tandem MS/MS profile (Figure 4.4A). However **28** shows presence of only one isomer as compared to the parent **10**, which contains two isomers. The hydroxyl group in the side chain of **28** forms a hydrogen bond with the carbonyls to stabilize the molecule and lock it in a single conformation. MS/MS profile of **28** also shows attachment of alcohol side chain (Figure 4.4B).

4.4.3 Structure Prediction using Chemical Shift (α_{H}) Difference

Following the preparation of CLs analogs **23**, **24**, **27** and **28**, it was important to investigate the occurrence of structural changes in the CL core structure. NMR spectroscopy was used as a tool to compare the structural similarity of the starting material **11** and **10** to those of their analogs (**23** and **24**) and (**27** and **28**) respectively (Figure 4.5). It was reported that chemical shifts of α protons of amino acids can be used to determine the extent, identity and location of secondary structural elements present in proteins (Wishart, et al., 1992).

Initially, the chemical shift of each amino acid in randomly coiled peptides was studied to determine the chemical shift index. The empirically derived chemical shift index can then be compared to the experimentally measured value of chemical shift for the $\text{H}\alpha$ of each amino acid in the peptide chain. Wishart et al. developed rules that predict protein secondary structure. These rules are as follows: “(a) If the α -proton chemical shift is greater than the range given in Table 4.1 for that residue, mark a “1” beside it. (b) If the α -proton chemical shift is less than the range given in Table 4.1 for that residue, mark a “-1” beside it. (c) If the α -proton chemical shift is within the given range in Table 4.1 for that residue, mark a “0” beside it. Any “dense” grouping of four or more “-1’s” not interrupted by a “1” is a helix. Any “dense” grouping of three or more “1’s” not interrupted by a “-1” is a β -strand. All other regions are designated as coil”(Wishart, et al., 1992).

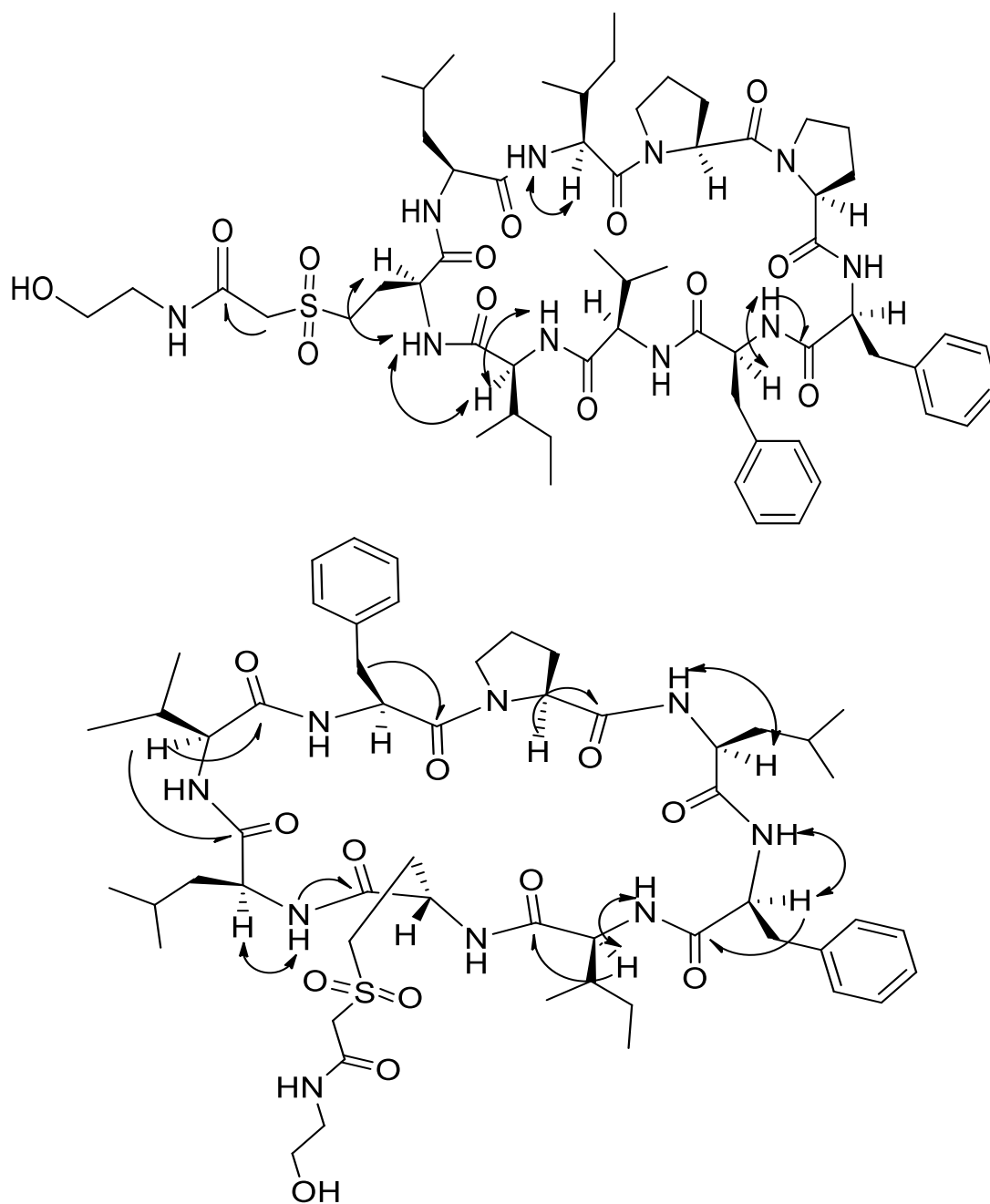


Figure 4.3 Structures of **24** and **28**. Double arrows show selected NOE correlations. Half arrows show selected HMBC correlations

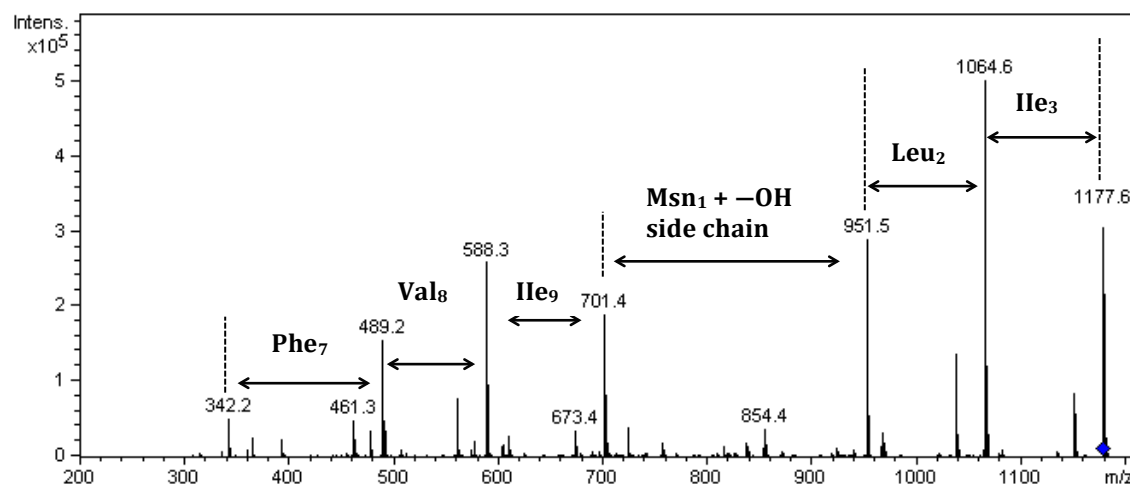
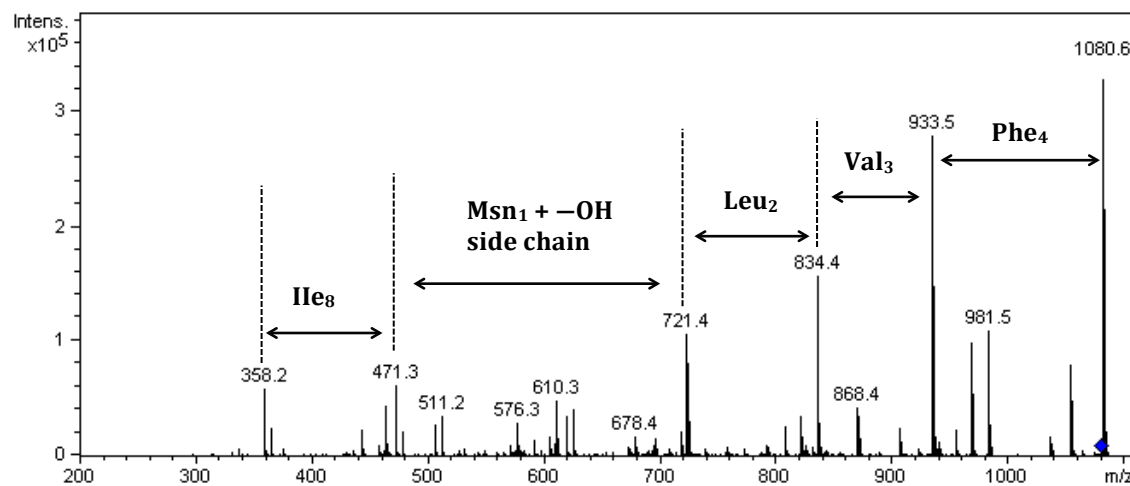
A**B**

Figure 4.4 CID MS/MS spectra of **24** (A) and **28** (B) showing fragmentation pattern

Table 4.1 Chemical shift values of α_{H} used in determination of secondary structure. Adapted from (Wishart, et al., 1992).

Residue	α_{H} range (ppm)	Residue	α_{H} range (ppm)
Ala	4.35 ± 0.10	Met	4.52 ± 0.10
Cys	4.65 ± 0.10	Asn	4.75 ± 0.10
Asp	4.76 ± 0.10	Pro	4.44 ± 0.10
Glu	4.29 ± 0.10	Gln	4.37 ± 0.10
Phe	4.66 ± 0.10	Arg	4.38 ± 0.10
Gly	3.97 ± 0.10	Ser	4.50 ± 0.10
His	4.63 ± 0.10	Thr	4.35 ± 0.10
Ile	3.95 ± 0.10	Val	3.95 ± 0.10
Lys	4.36 ± 0.10	Trp	4.70 ± 0.10
Leu	4.17 ± 0.10	Tyr	4.60 ± 0.10

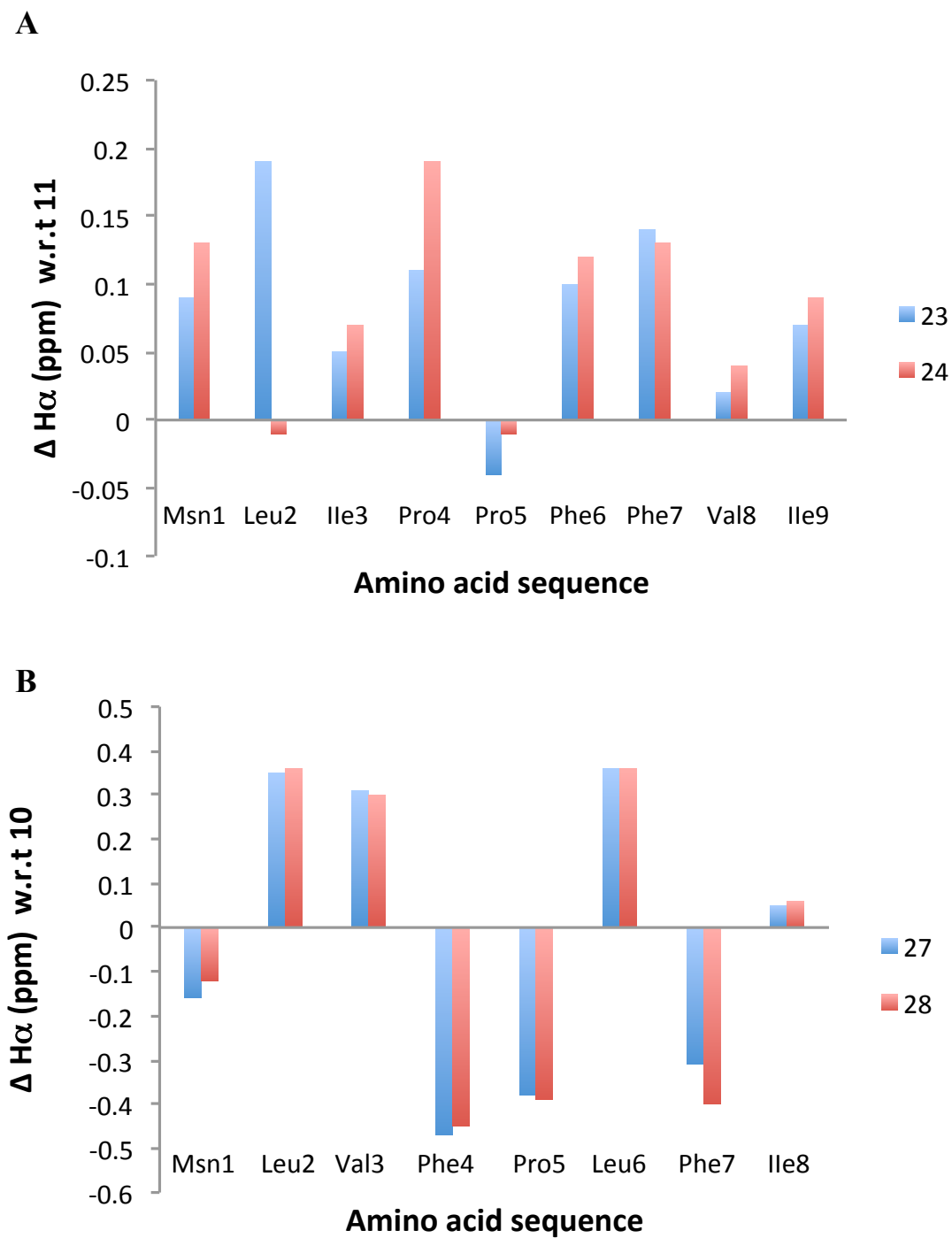


Figure 4.5 Chemical shift differences of α H signals for CLs **23** and **24** (A) and CLs **27** and **28** (B) compared with **11** and **10**, respectively

However this method is not a substitute for the two- and three-dimensional NMR methods, but it can complement them (Williamson & Asakura, 1997; Wishart, et al., 1995; Wishart, et al., 1992). Some of the examples to use chemical shifts of α proton of each amino acid residue to determine any structural changes in the backbone structure include Kalata B1 and α -Conotoxin ImI (Daly, et al., 2003; Dekan, et al., 2011). However, **27** and **28** shift perturbations indicated significant structural changes compared with **10**. The chemical shift difference of α H signals for CLs **23** and **24** compare to **11** for most of the residues (six out of nine for **23** and five out of nine amino acid residues for **24**) is less than 0.1. This indicates that substitution at the Msn side chain have might have effect on the structure of the molecule. But analogs **27** and **28** show major difference in Δ H α values compare to **10**, hence there might be structural difference between the parent and modified compounds. We have previously reported that **10** is present in two isomeric forms (Jadhav, et al., 2013). In contrast, **27** and **28** were present as single isomers. The stability of the single isomer may be due to hydrogen bonding of the amine and hydroxyl side chains. The chemical shift analysis showed that there might be structural changes induced by amine and alcohol side chain in **23** and **24**, but there are major structural changes in **27** and **28** compare to their parent CLs.

4.4.4 Experimental Procedures

CLB (2)

Sodium borohydride (1 eq, 0.07 g) and iodine (1.5 eq, 0.35 g) were added to a stirred solution of CL **3** (2 g, 1.9 mmol) in THF (25 mL). The resulting mixture was stirred at room temperature for 1h and was subsequently treated with water and was extracted with CH₂Cl₂. The solvent was removed and CL **2** was purified by flash column chromatography (1.8 g).

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Met¹: δ 4.27 (1H, dd, 8.4, 8.2, α), 1.99 (2H, m, β), 2.57 (1H, m, γ), 2.49 (1H, m, γ), 2.07 (3H, s, ϵ Me), 6.52 (1H, d, 6.4, NH); Leu²: δ 3.65 (1H, m, α), 1.63 (2H, m, β), 1.99 (1H, m, γ), 0.94 (3H, d, 6.6 Hz, δ Me), 0.92 (3H, d, 6.6 Hz, δ Me); Ile³: δ 4.44 (1H, m, α), 1.92 (1H, m, β), 1.78 (2H, m, γ), 1.05 (3H, d, 6.4 Hz, γ Me), 0.84 (3H, t, 7.5, δ Me), 7.34 (1H, br s, NH); Pro⁴: δ 4.03 (1H, m, α), 2.24 (1H, m, β), 1.92 (1H, m, β), 2.10 (1H, m, γ), 1.99 (1H, m, γ), 4.19 (1H, m, δ), 3.72 (1H, m, δ); Pro⁵: δ 3.94 (1H, m, α), 1.92 (2H, m, β), 1.50 (1H, m, γ), 0.74 (1H, m, γ), 3.34 (1H, m,

δ), 3.00 (1H, m, δ); Phe⁶: δ 4.74 (1H, m, α), 3.35 (1H, m, β), 3.00 (1H, m, β), 7.26 (m, 2H, δ), 7.18 (1H, m, ϵ), 7.10 (2H, m, ζ), 7.95 (1H, m, NH); Phe⁷: δ 3.95 (1H, m, α), 3.21 (1H, m, β), 2.79 (1H, m, β), 7.26 (m, 2H, δ), 7.18 (1H, m, ϵ), 7.10 (2H, m, ζ), 7.45 (1H, d, 6.4, NH); Val⁸: δ 3.89 (1H, m, α), 2.24 (1H, m, β), 1.05 (3H, d, 6.6 Hz, γ Me), 1.00 (3H, d, 6.6 Hz, γ Me), 8.09 (1H, br s, NH); Ile⁹: δ 4.20 (1H, m, α), 1.99 (1H, m, β), 1.50 (2H, m, γ), 0.95 (3H, d, 6.6 Hz, γ Me), 0.89 (3H, t, 7.5 Hz, δ Me), 6.82 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Met¹: δ 58.6 (α), 25.4 (β), 31.3 (γ), 15.3 (ϵ Me), 170.7 (C=O); Leu²: δ 58.6 (α), 28.9 (β), 25.2 (γ), 23.4 (δ Me), 21.4 (δ Me), 171.4 (C=O); Ile³: δ 56.2 (α), 37.2 (β), 25.4 (γ), 16.1 (γ Me), 11.0 (δ Me), 171.5 (C=O); Pro⁴: δ 60.9 (α), 31.5 (β), 21.5 (γ), 46.8 (δ), 170.0 (C=O); Pro⁵: δ 54.8 (α), 37.5 (β), 24.7 (γ), 48.3 (δ), 174.7 (C=O); Phe⁶: δ 60.9 (α), 36.0 (β), 137.2 (γ), 129.7 (δ), 129.0 (ϵ), 127.4 (ζ), 172.5 (C=O); Phe⁷: δ 55.6 (α), 37.7 (β), 136.4 (γ), 129.1 (δ), 128.9 (ϵ), 126.8 (ζ), 175.0 (C=O); Val⁸: δ 62.4 (α), 29.6 (β), 19.6 (γ Me), 18.3 (γ Me), 172.4 (C=O); Ile⁹: δ 54.0 (α), 35.0 (β), 25.4 (γ), 15.4 (γ Me), 10.8 (δ Me), 171.5 (C=O).

CLE' (13)

Similarly, CL **5** (2 g, 2.0 mmol) was reduced with sodium borohydride (1eq, 0.08 g) and iodine (1.5eq, 0.39 g) to obtain **13** (1.8 g) using the procedure above for CL **2**.

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Met¹: δ 4.69 (1H, m, α), 2.38 (1H, m, β), 1.83 (1H, m, β), 2.47 (2H, m, γ), 2.06 (3H, s, ϵ Me), 2.11 (3H, s, ϵ Me minor), 7.38 (1H, m, NH); Leu²: δ 4.25 (1H, m, α), 2.27 (1H, m, β), 1.81 (1H, m, β), 1.92 (1H, m, γ), 0.90 (3H, m, δ Me), 0.87 (3H, m, δ Me); Val³: δ 4.45 (1H, m, α), 2.07 (1H, m, β), 0.97 (3H, d, 6.4 Hz, γ Me), 0.94 (3H, d, 6.4 Hz, γ Me), 7.04 (1H, d, 7.5, NH); Phe⁴: δ 4.29 (1H, m, α), 3.00 (2H, m, β), 7.39-7.17 (m, 5H, δ , ϵ , ζ), 6.86 (1H, br s, NH); Pro⁵: δ 4.31 (1H, m, α), 1.65 (1H, m, β), 1.52 (1H, m, β), 1.66 (1H, m, γ), 1.46 (1H, m, γ), 3.37 (2H, m, δ); Leu⁶: δ 3.79 (1H, m, α), 2.21 (1H, m, β), 1.83 (1H, m, β), 1.57 (1H, m, γ), 0.94 (3H, m, δ Me), 0.90 (3H, m, δ Me), 7.59 (1H, m, NH); Phe⁷: δ 4.32 (1H, m, α), 3.16 (1H, m, β), 3.02 (1H, m, β), 7.39-7.17 (m, 5H, δ , ϵ , ζ), 7.57 (1H, m, NH); Ile⁸: δ 4.09 (1H, m, α), 1.83 (1H, m, β), 1.57 (2H, m, γ), 0.81 (3H, d, 6.4 Hz, γ Me), 0.87 (3H, m, δ Me), 6.07 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Met¹: δ 52.4 (α), 29.8 (β), 29.9 (γ), 15.5 (ϵ Me), 15.6 (ϵ Me (minor)), 171.1 (C=O); Leu²: δ 63.1 (α), 36.3 (β), 25.3 (γ), 23.4

(δ Me), 22.1 (δ Me), 172.1 (C=O); Val³: δ 57.5 (α), 31.7 (β), 19.4 (γ Me), 18.2 (γ Me), 171.7 (C=O); Phe⁴: δ 51.3 (α), 38.8 (β), 135.4 (γ), 129.2 (δ), 129.2 (ϵ), 127.6 (ζ), 171.4 (C=O); Pro⁵: δ 58.5 (α), 37.2 (β), 25.2 (γ), 46.5 (δ), 170.6 (C=O); Leu⁶: δ 55.3 (α), 30.9 (β), 25.1 (γ), 23.3 (δ Me), 21.4 (δ Me), 173.1 (C=O); Phe⁷: δ 53.4 (α), 37.4 (β), 135.8 (γ), 129.6 (δ), 129.5 (ϵ), 127.9 (ζ), 174.1 (C=O); Ile⁸: δ 60.3 (α), 35.8 (β), 25.2 (γ), 16.3 (γ Me), 11.7 (δ Me), 172.3 (C=O).

S^{4.1}-cyanomethylhomocysteine CLB (16)

CL **2** (50 mg, 0.05 mmol) was dissolved in *N*-methylpyrrolidone (2.5 mL) at room temperature. Diisopropylethylamine (20 μ L) and iodoacetonitrile (25 μ L) were sequentially added to the solution and the resulting mixture was heated to 100 °C for 20 h. The reaction mixture was extracted with EtOAc and the combined organic extract was washed with water and was subsequently concentrated under high vacuum (3 mbar) to yield a crude mixture. This crude mixture was purified using Sep-Pak cartridges and analyzed by HPLC. CL nitrile 16 was obtained (0.027 g).

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Met¹: δ 4.35 (1H, m, α), 2.41 (1H, m, β), 2.06 (1H, m, β), 2.86 (1H, m, γ), 2.78 (1H, m, γ), 3.28 (2H, s, SCH₂); Leu²: δ 3.81 (1H, m, α), 1.62 (2H, m, β), 2.16 (1H, m, γ), 0.95 (3H, d, 6.4 Hz, δ Me), 0.93 (3H, d, 6.4 Hz, δ Me); Ile³: δ 4.53 (1H, m, α), 1.92 (1H, m, β), 2.21 (2H, m, γ), 1.06 (3H, d, 6.8 Hz, γ Me), 0.89 (3H, t, 7.5, δ Me); Pro⁴: δ 3.98 (1H, m, α), 2.22 (1H, m, β), 1.91 (1H, m, β), 2.03 (1H, m, γ), 1.89 (1H, m, γ), 4.11 (1H, m, δ), 3.74 (1H, m, δ); Pro⁵: δ 4.02 (1H, m, α), 1.93 (2H, m, β), 1.51 (1H, m, γ), 0.87 (1H, m, γ), 3.34 (1H, m, δ), 3.06 (1H, m, δ); Phe⁶: δ 4.72 (1H, m, α), 3.16 (1H, m, β), 2.77 (1H, m, β), 7.24 (m, 3H, δ , ϵ), 7.07 (2H, m, ζ); Phe⁷: δ 4.23 (1H, m, α), 3.34 (2H, m, β), 7.30 (m, 2H, δ), 7.24 (1H, m, ϵ), 7.15 (2H, m, ζ), 7.96 (1H, d, 5, NH); Val⁸: δ 3.81 (1H, m, α), 2.19 (1H, m, β), 1.01 (3H, d, 7 Hz, γ Me), 0.98 (3H, d, 7 Hz, γ Me), 7.67 (1H, br s, NH); Ile⁹: δ 4.29 (1H, m, α), 2.06 (1H, m, β), 1.65 (2H, m, γ), 0.95 (3H, d, 6.4 Hz, γ Me), 0.87 (3H, t, 7.5 Hz, δ Me), 6.51 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Met¹: δ 53.4 (α), 25.2 (β), 28.9 (γ), 17.1 (SCH₂), 116.7 (CN), 170.8 (C=O); Leu²: δ 62.7 (α), 29.6 (β), 25.4 (γ), 23.4 (δ Me), 21.4 (δ Me), 171.6 (C=O); Ile³: δ 55.8 (α), 37.4 (β), 24.8 (γ), 15.3 (γ Me), 11.0 (δ Me), 172.0 (C=O); Pro⁴: δ 54.9 (α), 37.4 (β), 24.7 (γ), 48.3 (δ), 170.1 (C=O); Pro⁵: δ 61.0 (α), 31.6 (β),

21.7 (γ), 47.0 (δ), 174.6 (C=O); Phe⁶: δ 55.4 (α), 37.7 (β), 137.0 (γ), 129.6 (δ), 129.0 (ϵ), 127.4 (ζ), 172.2 (C=O); Phe⁷: δ 58.7 (α), 35.8 (β), 136.4 (γ), 129.1 (δ), 128.9 (ϵ), 127.0 (ζ), 175.1 (C=O); Val⁸: δ 58.6 (α), 29.6 (β), 19.5 (γ Me), 18.4 (γ Me), 172.2 (C=O); Ile⁹: δ 58.7 (α), 35.3 (β), 24.8 (γ), 16.2 (γ Me), 11.1 (δ Me), 172.1 (C=O).

1-2-aminobutanoic acid CLB (17)

CL **16** (30 mg, 0.03 mmol) was dissolved in dioxane: H₂O (3:1 v/v, 1.2 mL). Pd-C and LiOH were added to the reaction mixture that was stirred overnight at 140 °C under H₂ (1 atm). The reaction mixture was filtered and was neutralized with saturated NaHCO₃. The reaction mixture was extracted with ethyl acetate and was concentrated to obtain the crude product (35 mg) (**17**). HPLC-MS analyses indicated a [M+H]⁺ ion at m/z 1012.6275, corresponds to a molecular formula of C₅₅H₈₁N₉O₉.

S^{4,1}-cyanomethylhomocysteine S-dioxide CLC (18)

CL **16** (30 mg, 0.03 mmol) was dissolved in THF: H₂O (5:2 v/v, 1.2 mL) and was cooled to 0 °C before addition of aqueous solution of NaIO₄ (0.8 mL, excess). The reaction mixture was stirred overnight and was filtered. After removal of THF under reduced pressure, EtOAc was added to the resulting aqueous solution, which was then washed successively with water and brine. The organic layer was evaporated to yield a sulfoxide nitrile **18** (25 mg). HPLC-MS analyses indicated a [M+H]⁺ ion at m/z 1099.5958, corresponds to a molecular formula of C₅₇H₈₃N₁₀O₁₀S. The structure was further confirmed by LC-MS/MS analyses.

S^{4,1}-cyanomethylhomocysteine S, S-dioxide CLK (19)

CL **16** (9.4 mg, 0.009 mmol) was dissolved in dichloromethane (1 mL) to which *m*-CPBA (6.4 mg) was added. The reaction mixture was stirred at room temperature for 1 h, and was sequentially washed with a solution of 10% Na₂S₂O₃ (5 mL), saturated aq. NaHCO₃, and H₂O to yield the sulfonyl nitrile **19** (10 mg).

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Msn¹: δ 4.85 (1H, m, α), 2.69 (1H, m, β), 2.35 (1H, m, β), 3.67 (1H, m, γ), 3.53 (1H, m, γ), 4.04 (2H, s, SO₂CH₂), 7.35 (1H, m, NH); Leu²: δ 4.02 (1H, m, α), 2.12 (2H, m, β), 1.65 (1H, m, γ), 0.95 (3H, d, 6.4 Hz, δ Me), 0.93 (3H, d, 6.4 Hz, δ Me), 7.78 (1H, br s, NH); Ile³: δ 4.48 (1H, m, α), 1.93 (1H, m, β), 1.70 (2H, m, γ), 1.11 (3H, d, 6.4 Hz, γ Me), 0.87 (3H, m, δ Me), 8.13 (1H, br s, NH); Pro⁴: δ

3.87 (1H, m, α), 2.27 (1H, m, β), 1.70 (1H, m, β), 2.05 (1H, m, γ), 1.96 (1H, m, γ), 3.93 (1H, m, δ), 3.67 (1H, m, δ); Pro⁵: δ 4.12 (1H, m, α), 1.99 (2H, m, β), 1.47 (1H, m, γ), 1.24 (1H, m, γ), 3.35 (2H, m, δ); Phe⁶: δ 4.89 (1H, m, α), 3.04 (2H, dd, 4.2, 12.5 Hz, β), 7.26 (m, 3H, δ , ϵ), 7.16 (2H, m, ζ), 7.19 (1H, m, NH); Phe⁷: δ 4.07 (1H, m, α), 3.39 (2H, m, β), 7.34 (m, 2H, δ), 7.26 (1H, m, ϵ), 7.16 (2H, m, ζ), 7.61 (1H, br s, NH); Val⁸: δ 3.87 (1H, m, α), 2.12 (1H, m, β), 0.95 (3H, m, γ Me), 0.94 (3H, m, γ Me), 6.47 (1H, br s, NH); Ile⁹: δ 4.58 (1H, m, α), 1.99 (1H, m, β), 2.16 (2H, m, γ), 0.97 (3H, m, γ Me), 0.84 (3H, m, δ Me), 6.73 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 51.7 (α), 25.4 (β), 50.8 (γ), 42.5 (SO₂CH₂), 110.2 (CN), 171.1 (C=O); Leu²: δ 61.0 (α), 38.4 (β), 25.4 (γ), 22.9 (δ Me), 21.9 (δ Me), 171.1 (C=O); Ile³: δ 56.3 (α), 36.6 (β), 24.8 (γ), 15.6 (γ Me), 11.2 (δ Me), 171.8 (C=O); Pro⁴: δ 61.8 (α), 28.5 (β), 25.1 (γ), 48.1 (δ), 170.4 (C=O); Pro⁵: δ 61.0 (α), 32.1 (β), 21.9 (γ), 47.4 (δ), 173.8 (C=O); Phe⁶: δ 53.4 (α), 35.1 (β), 136.1 (γ), 129.7 (δ), 128.9 (ϵ), 127.5 (ζ), 173.8 (C=O); Phe⁷: δ 57.8 (α), 36.7 (β), 135.4 (γ), 129.1 (δ), 128.9 (ϵ), 127.3 (ζ), 175.1 (C=O); Val⁸: δ 59.1 (α), 29.3 (β), 19.6 (γ Me), 18.2 (γ Me), 173.0 (C=O); Ile⁹: δ 58.3 (α), 36.8 (β), 24.8 (γ), 16.1 (γ Me), 11.3 (δ Me), 171.8 (C=O).

S^{4.1}-(2-aminoethyl)homocysteine S, S-dioxide CLK (20)

CL **19** (8 mg, 0.007 mmol) was dissolved in acetic acid (0.5 mL) under argon and PtO₂ was added. The reaction mixture was stirred at RT under H₂ (1 atm) overnight. The reaction mixture was filtered and then neutralized with sat. NaHCO₃. The reaction mixture was extracted with ethyl acetate and was concentrated to obtain the crude product (10 mg) (**20**). HPLC-MS analyses indicated a [M+H]⁺ ion at m/z 1119.6275, corresponds to a molecular formula of C₅₇H₈₃N₁₀O₁₀S.

S^{4.1}-ethoxycarbonylmethylhomocysteine CLB (21)

CL **2** (0.3 g, 0.28 mmol) was dissolved in *N*-methylpyrrolidinone (10 mL) at room temperature. Diisopropylethylamine (2.5 eq, 0.12 mL) and iodoacetate (7 eq, 0.23 mL) were sequentially added to the solution and the resulting mixture was heated to 100 °C for 20 h. The reaction mixture was extracted with EtOAc and the combined organic extract was washed with water and was subsequently concentrated under high vacuum to yield a crude

mixture that was purified using Sep-Pak cartridges and analyzed by HPLC. The ester (-COOEt) derivative, **21**, was obtained in 0.27 g.

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Met¹: δ 4.27 (1H, dd, 8, 8.4 Hz, α), 1.95 (2H, m, β), 2.63 (1H, m, γ), 2.44 (1H, m, γ), 3.19 (2H, s, SCH₂), 4.16 (2H, q, 7Hz, SCH₂COOCH₂), 1.26 (3H, t, 7 Hz, S-CH₂COOCH₂CH₃), 6.44 (1H, br s, NH); Leu²: δ 3.61 (1H, m, α), 2.17 (2H, m, β), 1.98 (1H, m, γ), 0.93 (3H, d, 7 Hz, δMe), 0.91 (3H, d, 7 Hz, δMe); Ile³: δ 4.42 (1H, m, α), 1.91 (1H, m, β), 2.22 (2H, m, γ), 1.00 (3H, d, 6.6 Hz, γMe), 0.83 (3H, t, 7 Hz, δMe), 7.44 (1H, br s, NH); Pro⁴: δ 4.03 (1H, br s, α), 2.22 (1H, m, β), 1.91 (1H, m, β), 2.02 (1H, m, γ), 1.87 (1H, m, γ), 4.16 (1H, br s, δ), 3.71 (1H, m, δ); Pro⁵: δ 3.95 (1H, m, α), 1.95 (2H, m, β), 1.52 (1H, m, γ), 0.85 (1H, m, γ), 3.33 (1H, m, δ), 2.98 (1H, m, δ); Phe⁶: δ 4.71 (1H, m, α), 3.21 (1H, m, β), 2.77 (1H, m, β), 7.25 (m, 2H, δ), 7.19 (1H, m, ε), 7.09 (2H, m, ζ) 7.45 (1H, br s, NH); Phe⁷: δ 4.03 (1H, m, α), 3.36 (2H, m, β), 7.25 (m, 2H, δ), 7.19 (1H, m, ε), 7.09 (2H, m, ζ); Val⁸: δ 3.87 (1H, m, α), 2.22 (1H, m, β), 1.05 (3H, d, 7.3 Hz, γMe), 1.03 (3H, d, 7.3 Hz, γMe), 8.09 (1H, br s, NH); Ile⁹: δ 4.16 (1H, m, α), 2.10 (1H, m, β), 1.61 (2H, m, γ), 0.94 (3H, d, 7 Hz, γMe), 0.87 (3H, t, 7 Hz, δMe), 6.79 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Met¹: δ 58.1 (α), 25.4 (β), 29.8 (γ), 33.7 (SCH₂), 60.9 (SCH₂COOCH₂), 14.3 (SCH₂COOCH₂CH₃), 169.8 (COOCH₂CH₃), 170.7 (C=O); Leu²: δ 58.5 (α), 28.8 (β), 25.1 (γ), 23.5 (δMe), 21.3 (δMe), 171.2 (C=O); Ile³: δ 56.1 (α), 37.1 (β), 25.3 (γ), 16.1 (γMe), 11.1 (δMe), 171.5 (C=O); Pro⁴: δ 54.6 (α), 37.4 (β), 24.7 (γ), 48.3 (δ), 170.6 (C=O); Pro⁵: δ 61.5 (α), 31.5 (β), 21.5 (γ), 46.8 (δ), 174.8 (C=O); Phe⁶: δ 55.7 (α), 37.7 (β), 137.2 (γ), 129.6 (δ), 128.9 (ε), 127.4 (ζ), 172.4 (C=O); Phe⁷: δ 61.5 (α), 35.7 (β), 136.4 (γ), 129.0 (δ), 128.8 (ε), 126.7 (ζ), 175.1 (C=O); Val⁸: δ 62.4 (α), 29.5 (β), 19.6 (γMe), 18.3 (γMe), 172.4 (C=O); Ile⁹: δ 53.7 (α), 34.9 (β), 25.3 (γ), 15.2 (γMe), 10.7 (δMe), 172.0 (C=O).

S^{4,1}-ethoxycarbonylmethylhomocysteine CLE' (**25**)

Similarly, CL **13** (1 g, 1.0 mmol) was activated with iodoacetate (7 eq, 1.55g, 0.21 mL) and diisopropylethylamine (2.5 eq, 0.36 g, 0.45 mL) in *N*-methylpyrrolidone (8 mL) using above procedure to obtain **25** (1.1g)

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Met¹: δ 4.69 (1H, m, α), 2.45 (1H, m, β), 1.84 (1H, m, β), 2.62 (2H, m, γ), 3.19 (2H, s, SCH₂), 4.14 (2H, q, 7Hz, SCH₂COOCH₂), 1.25 (3H, t, 7 Hz, SCH₂COOCH₂CH₃), 7.42 (1H, d, 9, NH); Leu²: δ 4.31 (1H, m, α), 2.30 (1H, m, β), 1.75 (1H, m, β), 1.95 (1H, m, γ), 0.86 (6H, d, 6.6 Hz, δMe); Val³: δ 4.46 (1H, m, α), 2.06 (1H, m, β), 0.96 (3H, d, 6.4 Hz, γMe), 0.94 (3H, d, 6.4 Hz, γMe), 7.03 (1H, d, 8.6, NH); Phe⁴: δ 4.32 (1H, m, α), 3.01 (2H, m, β), 7.38-7.15 (m, 5H, δ, ε, ζ), 6.72 (1H, br s, NH); Pro⁵: δ 4.31 (1H, m, α), 1.57 (1H, m, β), 1.45 (1H, m, β), 1.64 (1H, m, γ), 1.50 (1H, m, γ), 3.37 (2H, m, δ); Leu⁶: δ 3.79 (1H, m, α), 2.22 (1H, m, β), 1.84 (1H, m, β), 1.57 (1H, m, γ), 0.87 (6H, m, δMe), 7.53 (1H, d, 7 Hz, NH); Phe⁷: δ 4.30 (1H, m, α), 3.16 (1H, m, β), 3.04 (1H, m, β), 7.38-7.15 (m, 5H, δ, ε, ζ), 7.59 (1H, br s, NH); Ile⁸: δ 4.11 (1H, m, α), 1.84 (1H, m, β), 1.57 (2H, m, γ), 0.80 (3H, d, 6.4 Hz, γMe), 0.87 (3H, m, δMe), 6.08 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Met¹: δ 52.2 (α), 31.1 (β), 29.6 (γ), 33.8 (SCH₂), 61.4 (SCH₂COOCH₂), 14.3 (SCH₂COOCH₂CH₃), 170.5 (SCH₂COOCH₂CH₃), 171.1 (C=O); Leu²: δ 51.3 (α), 29.9 (β), 30.8 (γ), 23.2 (δMe), 22.1 (δMe), 173.1 (C=O); Val³: δ 57.4 (α), 31.6 (β), 19.4 (γMe), 18.1 (γMe), 171.7 (C=O); Phe⁴: δ 53.3 (α), 38.8 (β), 135.4 (γ), 129.2 (δ), 129.1 (ε), 127.6 (ζ), 171.3 (C=O); Pro⁵: δ 63.2 (α), 38.2 (β), 25.3 (γ), 46.5 (δ), 170.8 (C=O); Leu⁶: δ 55.3 (α), 37.2 (β), 25.1 (γ), 22.8 (δMe), 21.9 (δMe), 173.1 (C=O); Phe⁷: δ 58.4 (α), 37.4 (β), 135.9 (γ), 129.6 (δ), 129.5 (ε), 127.9 (ζ), 174.1 (C=O); Ile⁸: δ 60.2 (α), 35.8 (β), 25.3 (γ), 16.3 (γMe), 11.7 (δMe), 172.3 (C=O).

S^{4,1}-ethoxycarbonylmethylhomocysteine S, S-dioxide CLK (**22**)

CL **21** (0.15 g, 0.13 mmol) was dissolved in dichloromethane (5 mL) to which *m*-CPBA (3eq, 0.1 g) was added. The reaction mixture was stirred at room temperature for 1 h, and was sequentially washed with a solution of 10% Na₂S₂O₃ (5 mL), saturated aq. NaHCO₃, and H₂O to yield Msn **22** (0.16 g).

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Msn¹: δ 4.61 (1H, m, α), 2.46 (2H, m, β), 3.46 (2H, m, γ), 3.95 (2H, s, SO₂CH₂), 4.23 (2H, q, 7Hz, SO₂CH₂COOCH₂), 1.29 (3H, t, 7 Hz, SO₂CH₂COOCH₂CH₃), 7.20 (1H, d, 7, NH); Leu²: δ 4.06 (1H, d, 7.9 Hz, α), 2.16 (2H, m, β), 1.63 (1H, m, γ), 0.95 (3H, d, 6.4 Hz, δMe), 0.93 (3H, d, 6.4 Hz, δMe), 7.63 (1H, d, 6 Hz, NH); Ile³: δ 4.48 (1H, m, α), 1.93 (1H, m, β), 1.50 (2H, m, γ), 1.07 (3H, d,

6.8 Hz, γ Me), 0.84 (3H, t, 6.8 Hz, δ Me), 6.60 (1H, br s, NH); Pro⁴: δ 3.81 (1H, m, α), 2.16 (1H, m, β), 1.67 (1H, m, β), 2.17 (1H, m, γ), 1.95 (1H, m, γ), 4.01 (1H, m, δ), 3.46 (1H, m, δ); Pro⁵: δ 4.06 (1H, m, α), 2.16 (1H, m, β), 1.94 (1H, m, β), 1.49 (1H, m, γ), 0.96 (1H, m, γ), 3.29 (2H, m, δ); Phe⁶: δ 4.85 (1H, m, α), 3.09 (2H, dd, 4.2, 12.5 Hz, β), 7.24 (m, 2H, δ), 7.20 (1H, m, ϵ), 7.12 (2H, m, ζ); Phe⁷: δ 3.99 (1H, m, α), 3.67 (2H, m, β), 7.30 (m, 3H, δ , ϵ), 7.12 (2H, m, ζ); 7.72 (1H, br s, NH); Val⁸: δ 3.87 (1H, m, α), 2.16 (1H, m, β), 0.95 (3H, d, 6.4 Hz, γ Me), 0.93 (3H, d, 6.4 Hz, γ Me); Ile⁹: δ 4.48 (1H, m, α), 1.95 (1H, m, β), 2.16 (2H, m, γ), 0.92 (3H, d, 6.4 Hz, γ Me), 0.90 (3H, t, 6.4 Hz, δ Me).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 52.4 (α), 25.3 (β), 51.2 (γ), 57.6 (SO₂CH₂), 62.7 (SO₂CH₂COOCH₂), 14.1 (SO₂CH₂COOCH₂CH₃), 162.8 (COOCH₂CH₃), 170.6 (C=O); Leu²: δ 61.0 (α), 37.9 (β), 25.4 (γ), 23.2 (δ Me), 21.7 (δ Me), 171.4 (C=O); Ile³: δ 56.3 (α), 36.2 (β), 24.8 (γ), 15.6 (γ Me), 11.1 (δ Me), 171.7 (C=O); Pro⁴: δ 62.0 (α), 28.6 (β), 25.1 (γ), 48.1 (δ), 170.5 (C=O); Pro⁵: δ 56.6 (α), 31.9 (β), 21.7 (γ), 47.1 (δ), 174.1 (C=O); Phe⁶: δ 54.2 (α), 36.1 (β), 136.4 (γ), 129.8 (δ), 129.0 (ϵ), 127.4 (ζ), 173.0 (C=O); Phe⁷: δ 58.3 (α), 36.2 (β), 136.3 (γ), 128.9 (δ), 127.3 (ϵ), 127.3 (ζ), 175.2 (C=O); Val⁸: δ 58.8 (α), 29.4 (β), 19.6 (γ Me), 18.3 (γ Me), 172.8 (C=O); Ile⁹: δ 58.3 (α), 36.7 (β), 25.0 (γ), 16.1 (γ Me), 11.3 (δ Me), 171.8 (C=O).

S^{4,1}-ethoxycarbonylmethylhomocysteine S, S-dioxide CLJ (26)

Similarly, CL **25** (0.7 g, 0.68 mmol) was oxidized with *m*-CPBA (3eq, 0.35 g) in dichloromethane (15 mL) using the procedure above to obtain **26** (0.74 g).

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Msn¹: δ 4.71 (1H, m, α), 2.58 (1H, m, β), 2.18 (1H, m, β), 3.52 (1H, m, γ), 3.17 (1H, m, γ), 3.92 (2H, dd, 14.6, 14.6, SO₂CH₂), 4.23 (2H, q, 7Hz, SO₂CH₂COOCH₂), 1.29 (3H, t, 7 Hz, SO₂CH₂COOCH₂CH₃), 7.69 (1H, br s, NH); Leu²: δ 4.33 (1H, m, α), 2.32 (1H, m, β), 1.72 (1H, m, β), 1.95 (1H, m, γ), 0.88 (3H, m, δ Me), 0.81 (3H, m, δ Me), 6.83 (1H, br s, NH); Val³: δ 4.47 (1H, m, α), 2.05 (1H, m, β), 0.97 (3H, d, 6.8 Hz, γ Me), 0.94 (3H, d, 6.8 Hz, γ Me), 6.97 (1H, br s, NH); Phe⁴: δ 4.34 (1H, m, α), 3.02 (2H, m, β), 7.39-7.18 (m, 5H, δ , ϵ , ζ), 6.66 (1H, br s, NH); Pro⁵: δ 4.12 (1H, m, α), 1.72 (1H, m, β), 1.42 (1H, m, β), 1.65 (1H, m, γ), 1.47 (1H, m, γ), 3.36 (2H, m, δ); Leu⁶: δ 3.79 (1H, m, α), 2.18 (1H, m, β), 1.86 (1H, m, β), 1.54 (1H, m, γ), 0.94 (3H, d, 6.8 Hz, δ Me), 0.88 (3H, m, δ Me), 7.61 (1H, d, 7 Hz, NH); Phe⁷: δ 4.31 (1H, m, α),

3.17 (1H, m, β), 3.07 (1H, m, β), 7.39-7.18 (m, 5H, δ , ϵ , ζ), 7.69 (1H, br s, NH); Ile⁸: δ 4.07 (1H, m, α), 1.86 (1H, m, β), 1.54 (2H, m, γ), 0.79 (3H, m, γ Me), 0.86 (3H, m, δ Me), 6.12 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 51.3 (α), 24.8 (β), 51.4 (γ), 57.5 (SO₂CH₂), 62.7 (SO₂CH₂COOCH₂), 14.1 (SO₂CH₂COOCH₂CH₃), 162.9 (SO₂CH₂COOCH₂CH₃), 170.9 (C=O); Leu²: δ 51.2 (α), 37.0 (β), 25.4 (γ), 23.3 (δ Me), 21.8 (δ Me), 171.6 (C=O); Val³: δ 57.5 (α), 32.0 (β), 19.4 (γ Me), 18.2 (γ Me), 171.6 (C=O); Phe⁴: δ 53.1 (α), 38.9 (β), 135.5 (γ), 129.2 (δ), 129.1 (ϵ), 127.6 (ζ), 171.2 (C=O); Pro⁵: δ 54.2 (α), 31.0 (β), 25.2 (γ), 46.5 (δ), 170.4 (C=O); Leu⁶: δ 55.3 (α), 29.9 (β), 29.3 (γ), 23.0 (δ Me), 21.4 (δ Me), 173.4 (C=O); Phe⁷: δ 58.4 (α), 37.3 (β), 135.6 (γ), 129.6 (δ), 129.5 (ϵ), 127.9 (ζ), 174.6 (C=O); Ile⁸: δ 60.2 (α), 35.7 (β), 25.0 (γ), 16.3 (γ Me), 11.7 (δ Me), 172.5 (C=O).

S^{4.1}-[2-(2-aminoethylamino)-2-oxo-ethyl]homocysteine S, S-dioxide CLK (23)

CL **22** (0.1 g, 0.09 mmol) was dissolved in methanol and ethylene diamine (excess) was added. The resulting reaction mixture was refluxed overnight. The organic solvents were evaporated and the resulting crude product was purified using Sep-Pak cartridges and was analyzed by HPLC. CL **23** was obtained in 0.09 g.

¹HNMR (500 MHz, CDCl₃, COSY, NOESY): Msn¹: δ 4.72 (1H, m, α), 2.50 (1H, m, β), 2.40 (1H, m, β), 3.48 (1H, m, γ), 3.41 (1H, m, γ), 3.90 (2H, s, SO₂CH₂), 2.87 (4H, m, NHCH₂CH₂NH₂), 7.41 (1H, br s, NH); Leu²: δ 3.88 (1H, br s, α), 2.17 (2H, br s, β), 1.64 (1H, m, γ), 0.92 (6H, d, 6.6 Hz, δ Me), 7.75 (1H, br s, NH); Ile³: δ 4.47 (1H, m, α), 1.92 (1H, m, β), 1.50 (2H, m, γ), 1.08 (3H, d, 6.6 Hz, γ Me), 0.86 (3H, t, 7 Hz, δ Me), 8.03 (1H, br s, NH); Pro⁴: δ 3.79 (1H, br s, α), 2.17 (1H, m, β), 1.64 (1H, m, β), 2.04 (1H, m, γ), 1.94 (1H, m, γ), 3.97 (1H, br s, δ), 3.65 (1H, m, δ); Pro⁵: δ 4.11 (1H, m, α), 2.12 (1H, m, β), 1.95 (1H, m, β), 1.50 (1H, m, γ), 0.96 (1H, m, γ), 3.31 (2H, m, δ); Phe⁶: δ 4.86 (1H, m, α), 3.10 (1H, m, β), 3.00 (1H, m, β), 7.24 (m, 3H, δ , ϵ), 7.15 (2H, m, ζ); Phe⁷: δ 3.97 (1H, m, α), 3.31 (2H, m, β), 7.32 (m, 2H, δ), 7.24 (1H, m, ϵ), 7.15 (2H, m, ζ), 7.67 (1H, br s, NH); Val⁸: δ 3.88 (1H, m, α), 2.17 (1H, m, β), 0.92 (6H, d, 6.6 Hz, γ Me); Ile⁹: δ 4.53 (1H, m, α), 1.98 (1H, m, β), 1.64 (2H, m, γ), 0.96 (3H, d, 6.4 Hz, γ Me), 0.84 (3H, t, 6.4 Hz, δ Me), 6.78 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 57.2 (α), 25.1 (β), 50.7 (γ), 58.6 (SO₂CH₂), 42.9 (NHCH₂CH₂NH₂), 41.2 (NHCH₂CH₂NH₂), 171.9 (C=O); Leu²: δ 61.8 (α), 29.9 (β), 25.5 (γ), 23.1 (δMe), 21.9 (δMe), 171.9 (C=O); Ile³: δ 56.3 (α), 36.7 (β), 25.4 (γ), 15.6 (γMe), 11.2 (δMe), 171.9 (C=O); Pro⁴: δ 59.1 (α), 28.7 (β), 25.1 (γ), 48.2 (δ), 170.9 (C=O); Pro⁵: δ 61.2 (α), 32.0 (β), 21.7 (γ), 47.3 (δ), 173.6 (C=O); Phe⁶: δ 54.2 (α), 35.6 (β), 136.4 (γ), 129.8 (δ), 129.0 (ε), 127.4 (ζ), 173.6 (C=O); Phe⁷: δ 61.2 (α), 38.4 (β), 135.9 (γ), 129.1 (δ), 128.9 (ε), 127.3 (ζ), 175.2 (C=O); Val⁸: δ 59.1 (α), 29.4 (β), 19.7 (γMe), 18.3 (γMe), 173.2 (C=O); Ile⁹: δ 58.3 (α), 36.7 (β), 25.4 (γ), 16.1 (γMe), 11.3 (δMe), 173.0 (C=O).

S^{4.1}-[2-(2-aminoethylamino)-2-oxo-ethyl]homocysteine S, S-dioxide CLJ (27)

Similarly, CL **26** (0.3 g, 0.29 mmol) was coupled with ethylene diamine (excess) in methanol (6 mL) to obtain **27** (0.27 g) using the procedure described above.

¹H NMR (500 MHz, CDCl₃, COSY, NOESY) Msn¹: δ 4.92 (1H, m, α), 2.58 (1H, m, β), 2.20 (1H, m, β), 3.43 (2H, m, γ), 3.41 (2H, m, SO₂CH₂), 3.06 (2H, m, NHCH₂CH₂NH₂), 3.43 (2H, m, NHCH₂CH₂ NH₂), 4.36 (2H, br s, NHCH₂CH₂NH₂), 6.81 (1H, m, NH); Leu²: δ 4.01 (1H, m, α), 1.74 (1H, m, β), 1.65 (1H, m, β), 2.38 (1H, m, γ), 0.84 (3H, d, 6.6 Hz, δMe), 0.78 (3H, d, 6.6 Hz, δMe), 8.97 (1H, br s, NH); Val³: δ 4.19 (1H, m, α), 2.38 (1H, m, β), 0.95 (6H, m, γMe), 6.49 (1H, br s, NH); Phe⁴: δ 4.81 (1H, m, α), 3.22 (1H, m, β), 3.12 (1H, m, β), 7.25-7.06 (m, 5H, δ, ε, ζ), 7.02 (1H, m, NH); Pro⁵: δ 4.47 (1H, m, α), 2.38 (1H, m, β), 1.75 (1H, m, β), 2.04 (1H, m, γ), 1.95 (1H, m, γ), 3.73 (1H, m, δ), 3.52 (1H, m, δ); Leu⁶: δ 3.45 (1H, m, α), 2.05 (1H, m, β), 1.95 (1H, m, β), 1.75 (1H, m, γ), 0.92 (6H, m, δMe), 8.11 (1H, m, NH); Phe⁷: δ 5.24 (1H, m, α), 3.07 (2H, m, β), 7.25-7.06 (m, 5H, δ, ε, ζ), 7.52 (1H, br s, NH); Ile⁸: δ 4.08 (1H, m, α), 1.28 (1H, m, β), 1.46 (1H, m, γ), 1.41 (1H, m, γ), 1.04 (3H, m, γMe), 0.91 (3H, m, δMe).

¹³C NMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 50.3 (α), 25.0 (β), 50.8 (γ), 60.4 (SO₂CH₂), 40.4 (NHCH₂CH₂NH₂), 40.7 (NHCH₂CH₂NH₂), 163.1 (CONHCH₂CH₂NH₂), 170.5 (C=O); Leu²: δ 56.4 (α), 39.8 (β), 29.6 (γ), 23.0 (δMe), 22.3 (δMe), 172.9 (C=O); Val³: δ 59.7 (α), 30.5 (β), 19.6 (γMe), 17.6 (γMe), 171.8 (C=O); Phe⁴: δ 54.5 (α), 36.7 (β), 138.0 (γ), 129.0 (δ), 128.7 (ε), 126.7 (ζ), 172.9 (C=O); Pro⁵: δ 63.7 (α), 29.8 (β), 25.0 (γ), 48.4 (δ), 173.9 (C=O); Leu⁶: δ 59.7 (α), 25.6 (β), 25.0 (γ), 22.5 (δMe),

22.0 (δ Me), 173.4 (C=O); Phe⁷: δ 50.8 (α), 35.9 (β), 137.3 (γ), 128.8 (δ), 128.3 (ϵ), 126.6 (ζ), 172.3 (C=O); Ile⁸: δ 54.5 (α), 25.3 (β), 39.8 (γ), 17.6 (γ Me), 10.2 (δ Me), 172.3 (C=O).

S^{4.1}-[2-(2-hydroxyethylamino)-2-oxo-ethyl]homocysteine S, S-dioxide CLK (24)

CL **22** (90 mg, 0.08 mM) was dissolved in methanol (5 mL) and ethanolamine (100 μ L, excess) was added. The resulting reaction mixture was refluxed overnight. The organic solvents were evaporated and the resulting crude product was analyzed by reversed-phase HPLC and purified using preparative HPLC. CL **24** was obtained in 55 mg yield.

¹HNMR (500 MHz, CDCl₃, COSY, NOESY) Msn¹: δ 4.68 (1H, m, α), 2.45 (2H, m, β), 3.45 (2H, m, γ), 3.94 (2H, s, SO₂CH₂), 3.71 (2H, m, NHCH₂CH₂OH), 3.54 (1H, m, NHCH₂CH₂OH), 3.31 (1H, m, NHCH₂CH₂OH), 7.41 (1H, br s, NH); Leu²: δ 4.08 (1H, br s, α), 2.14 (2H, m, β), 1.63 (1H, m, γ), 0.91 (6H, d, 6.4 Hz, δ Me), 7.74 (1H, br s, NH); Ile³: δ 4.45 (1H, m, α), 1.93 (1H, m, β), 1.48 (2H, m, γ), 1.07 (3H, d, 6.6 Hz, γ Me), 0.85 (3H, m, δ Me), 7.99 (1H, br s, NH); Pro⁴: δ 3.71 (1H, m, α), 2.14 (1H, m, β), 1.62 (1H, m, β), 2.05 (1H, m, γ), 1.92 (1H, m, γ), 3.94 (1H, m, δ), 3.67 (1H, m, δ); Pro⁵: δ 4.08 (1H, m, α), 1.92 (2H, m, β), 1.48 (1H, m, γ), 0.96 (1H, m, γ), 3.31 (2H, m, δ); Phe⁶: δ 4.84 (1H, m, α), 3.1 (1H, m, β), 2.98 (1H, m, β), 7.25 (m, 3H, δ , ϵ), 7.14 (2H, m, ζ); Phe⁷: δ 3.98 (1H, m, α), 3.67 (2H, m, β), 7.30 (m, 2H, δ), 7.25 (1H, m), 7.14 (2H, m, ζ), 7.64 (1H, m, NH); Val⁸: δ 3.86 (1H, m, α), 1.92 (1H, m, β), 0.91 (6H, d, 7 Hz, γ Me); Ile⁹: δ 4.51 (1H, m, α), 1.93 (1H, m, β), 1.63 (2H, m, γ), 0.96 (3H, d, 6.6 Hz, γ Me), 0.85 (3H, m, δ Me), 6.77 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 52.4 (α), 24.8 (β), 50.6 (γ), 58.5 (SO₂CH₂), 61.6 (NHCH₂CH₂OH), 43.2 (NHCH₂CH₂OH), 161.6 (CONHCH₂CH₂OH), 171.1 (C=O); Leu²: δ 58.8 (α), 38.4 (β), 25.4 (γ), 23.1 (δ Me), 21.9 (δ Me), 171.1 (C=O); Ile³: δ 56.3 (α), 36.6 (β), 25.2 (γ), 15.5 (γ Me), 11.1 (δ Me), 172.0 (C=O); Pro⁴: δ 56.8 (α), 28.7 (β), 25.1 (γ), 48.2 (δ), 170.8 (C=O); Pro⁵: δ 61.2 (α), 31.9 (β), 21.7 (γ), 47.3 (δ), 173.7 (C=O); Phe⁶: δ 54.4 (α), 35.7 (β), 136.4 (γ), 129.8 (δ), 129.0 (ϵ), 127.3 (ζ), 170.8 (C=O); Phe⁷: δ 59.0 (α), 38.4 (β), 136.0 (γ), 129.1 (δ), 129.0 (ϵ), 127.3 (ζ), 175.2 (C=O); Val⁸: δ 62.0 (α), 29.4 (β), 19.7 (γ Me), 18.3 (γ Me), 172.9 (C=O); Ile⁹: δ 58.1 (α), 36.6 (β), 25.4 (γ), 16.0 (γ Me), 11.2 (δ Me), 171.4 (C=O).

S^{4.1}-[2-(2-hydroxyethylamino)-2-oxo-ethyl]homocysteine S, S-dioxide CLJ (28)

Using the procedure described above CL **26** (50 mg, 0.05 mM) was reacted with ethanolamine (100 μ L, excess) in methanol (3 mL) to obtain **28** (33 mg).

^1H NMR (500 MHz, CDCl_3 , COSY, NOESY) Msn¹: δ 4.88 (1H, m, α), 2.57 (1H, m, β), 2.19 (1H, m, β), 3.45 (2H, m, γ), 4.00 (1H, m, SO_2CH_2), 3.89 (1H, m, SO_2CH_2), 4.48 (1H, m, $\text{NHCH}_2\text{CH}_2\text{OH}$), 3.51 (1H, m, $\text{NHCH}_2\text{CH}_2\text{OH}$), 3.89 (1H, m, $\text{NHCH}_2\text{CH}_2\text{OH}$), 3.20 (1H, m, $\text{NHCH}_2\text{CH}_2\text{OH}$), 7.75 (1H, br s, $\text{NHCH}_2\text{CH}_2\text{OH}$), 6.83 (1H, m, NH); Leu²: δ 4.00 (1H, m, α), 1.62 (2H, m, β), 2.35 (1H, m, γ), 0.85 (3H, d, 6.6 Hz, δMe), 0.78 (3H, d, 6.6 Hz, δMe), 9.17 (1H, br s, NH); Val³: δ 4.20 (1H, m, α), 2.48 (1H, m, β), 0.94 (6H, m, γMe), 6.24 (1H, br s, NH); Phe⁴: δ 4.79 (1H, m, α), 3.34 (1H, m, β), 3.09 (1H, m, β), 7.25–7.06 (m, 5H, δ , ϵ , ζ), 7.02 (1H, m, NH); Pro⁵: δ 4.48 (1H, m, α), 2.38 (1H, m, β), 1.78 (1H, m, β), 2.07 (1H, m, γ), 1.97 (1H, m, γ), 3.75 (1H, m, δ), 3.51 (1H, m, δ); Leu⁶: δ 3.45 (1H, m, α), 2.38 (2H, m, β), 1.68 (1H, m, γ), 0.92 (6H, m, δMe), 8.13 (1H, d, 7.5, NH); Phe⁷: δ 5.33 (1H, m, α), 3.75 (1H, m, β), 3.09 (1H, m, β), 7.25–7.06 (m, 5H, δ , ϵ , ζ), 7.49 (1H, d, 9.9, NH); Ile⁸: δ 4.07 (1H, m, α), 1.26 (1H, m, β), 1.49 (1H, m, γ), 1.39 (1H, m, γ), 1.09 (3H, d, 6.4 Hz, γMe), 0.91 (3H, m, δMe).

^{13}C NMR (125 MHz, CDCl_3 , HMQC, HMBC): Msn¹: δ 50.2 (α), 25.0 (β), 50.3 (γ), 60.9 (SO_2CH_2), 61.2 ($\text{NHCH}_2\text{CH}_2\text{OH}$), 43.7 ($\text{NHCH}_2\text{CH}_2\text{OH}$), 163.0 ($\text{CONHCH}_2\text{CH}_2\text{OH}$), 170.8 (C=O); Leu²: δ 56.5 (α), 40.6 (β), 29.5 (γ), 22.9 (δMe), 22.4 (δMe), 173.1 (C=O); Val³: δ 59.3 (α), 32.4 (β), 19.6 (γMe), 17.4 (γMe), 171.8 (C=O); Phe⁴: δ 54.6 (α), 36.3 (β), 138.4 (γ), 129.0 (δ), 128.8 (ϵ), 126.6 (ζ), 173.2 (C=O); Pro⁵: δ 63.8 (α), 30.5 (β), 25.1 (γ), 48.4 (δ), 174.0 (C=O); Leu⁶: δ 59.7 (α), 25.7 (β), 24.9 (γ), 22.4 (δMe), 22.1 (δMe), 173.6 (C=O); Phe⁷: δ 50.8 (α), 35.8 (β), 137.2 (γ), 128.7 (δ), 128.4 (ϵ), 126.6 (ζ), 172.3 (C=O); Ile⁸: δ 54.7 (α), 25.2 (β), 39.8 (γ), 17.7 (γMe), 10.0 (δMe), 172.2 (C=O).

4.5 Conclusion

In summary, we have described methods to modify CLs (**3** and **5**) using methionine as point of attachment to produce **23**, **24**, **27** and **28**. The simplicity and the operational ease make these methods suitable for producing CL analogs to be used for different applications. The structures of all derivatives (ester, nitrile, amine and alcohol) were elucidated using high-resolution HPLC-MS-ESI, tandem MS, 1D (^1H and ^{13}C) and 2D (NOE, COSY, and HMBC) NMR spectroscopic methods. This is the first report on their complete characterization. The similarity of NMR chemical shifts of **23** and **24** with parent CL **11** for most of the amino acid residues shows that the modified CLs may have similar overall structure as the parent CL. However, **27** and **28** shows major shift perturbations when compared to the starting material **10**.

4.6 Brief Introduction to Chapter 5

Biochemical and biophysical studies of molecules and their interactions often requires covalent coupling where such coupling enables the elucidation of specific molecular processes. Flax CLs **2** and **13**, as described in Chapter 4, were modified to form stable Msn analogs of **10** and **11** containing amino and hydroxyl side chain. The introduced reactive side chains are now readily covalently coupled with the functional groups of molecules (e.g. fluorescence labels), macromolecules (BSA) and affinity chromatography media for further studies. In Chapter 5, for example, these modified CLs were covalently linked to fluorescent molecules yielding novel fluorescent CLs. As a prerequisite of utilizing the CL-coumarin fluorescent label the absorption and fluorescence spectra of CL-coumarin complexes in solvents were studied. CLs could be used in charge transfer devices and also in electro optical devices. CLs were also coupled with solid affinity matrix and resulting CL matrix was used to purify apolipoprotein A1 from chicken serum. CLs were covalently coupled to BSA and these were used to induce the production of polyclonal antibodies in rabbits. The resulting polyclonal antibodies (pAbs) were used to develop a competitive indirect enzyme-linked immunosorbent assay (CI-ELISA).

CHAPTER 5

APPLICATIONS OF FLAX CYCLOLINOPEPTIDES

5.1 Abstract

Synthetic analogs of flax CLs **10** and **11**, containing reactive $-\text{NH}_2$ and $-\text{OH}$ groups, were selectively coupled with complementary functional groups present in fluorescent labels, affinity resin and protein (BSA). Amino analogs were coupled with the *N*-hydroxysuccinimide ester of coumarin dye and affinity matrix to form a covalent linkage. We have developed a general synthetic protocol that may be used to prepare various fluorescent CL derivatives from commercially available dyes. In this study *N*-hydroxysuccinimide coumarin dye was covalently coupled with amine CLs. The spectroscopic analysis was performed in different solvents such as methanol, ethanol, acetonitrile and DMF. The alcoholic solvents have shown better quantum yields compare to other solvents. CLs were also used as affinity tag to capture high-density lipoprotein (HDL) from the chicken serum. The agarose gel matrix containing activating group such as *N*-hydroxysuccinimide was coupled with amine CL and this CL-matrix was used to isolate protein from serum. The sequence of the isolated protein with MW of 28 kDa is identical to apolipoprotein A1 (Apo A1). In addition, CLs containing hydroxyl side chain were used as haptens. The haptens were conjugated to BSA through carbonyldiimidazole coupling and the incorporation of haptens was compared by MALDI, LC-ESI-MS, and SDS-PAGE and polyclonal antibodies were produced in rabbits. The polyclonal antibodies can be used both for the therapeutic purpose and detection of CLs in flax and flaxseed samples. Competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) was developed by the use of specific polyclonal antibodies (pAbs).

5.2 Introduction

Cyclic peptides are important class of structurally diverse complex molecules that exhibits a wide range of biological activity. Peptides have been identified that are antimicrobial, antiviral, insecticidal, anti-inflammatory and immunosuppressant. Cyclic peptides are typically much more stable when compared to linear peptides. They resist proteolytic degradation, due to their conformational restriction, and lack both C and N-terminus, making them stable to exopeptidase. The increase in rigidity of these structurally constrained peptides makes them suitable for selective binding and ligands for macromolecular receptors. Selective transformation of functional groups on biomolecules such as carbohydrates and peptides is important in biomedical research to prepare analogues and study structure-function relationships. In addition, tagged peptides were also used in forming fluorescent adducts, affinity media, isotope labeled peptides and haptens for antibody production (Bode, et al., 2012; Hamamoto, et al., 2011; Jayaraj, et al., 2003; Tozzi, et al., 2003).

CLA (**1**) a natural nonapeptide isolated from linseed oil in 1959 by Kaufmann and coworker (Kaufmann & Tobschirbel, 1959). Another eight cyclic nona- and octapeptides, CLs (**2–9**) were also isolated from the seeds of *L. usitatissimum* L. and their structures were determined by extensive 2D nuclear magnetic resonance (NMR) methods and chemical degradation (Matsumoto, et al., 2001; Morita, et al., 1999) (Figure 2.6 and Table 2.3, Chapter 2). In addition **11** and **10** were chemically synthesized from their parent CLs **3** and **5**, respectively and they were also be obtained from ageing flaxseed (Jadhav, et al., 2013; Matsumoto, et al., 2001). **1** was found to possess the immunosuppressive activity comparable to that of CsA (Wieczorek, et al., 1991). The strong immunosuppressive activity of **1** makes the CL interesting from the point of view of its possible utilization in medicine (Wieczorek, et al., 1991). Of all the eleven CLs, **1** showed the best immunosuppressive activity on human peripheral blood lymphocytes. It showed the inhibitory effects on concanavalin A with an IC₅₀ of 2.5 µg/mL followed by **11** (25.2 µg/mL) and **10** (28.1 µg/mL) (Morita & Takeya, 2010). The molecular mechanism of **1** and CsA is similar, i.e. first they bind with cyclophilin, and then inhibits the phosphatase calcineurin (Siemion, et al., 1999). The biological activity and presence of hydrophobic amino acid residues makes them good candidates in the fields of fluorescence labeling, affinity purification and antibody production. The attachment of fluorescence tag and

production of antibodies against these CLs will help in monitoring these bioactive CLs *in vitro* and *in vivo*.

In this study, the CLs were systematically linked to molecules or materials of interest such as fluorescence tags, affinity resin and proteins. In addition, these CL conjugates were characterized using spectrometric techniques including NMR, MS/MS, MALDI, ESI, and SDS-PAGE. This study helps to generate novel CL conjugates coupled to different set of molecules such as big molecule (protein), small molecule (fluorescent dye) and surface (matrix). The use of hydrophobic CL in different applications will help to increase its utility in addition to its biological activity.

5.3 Materials and Methods

5.3.1 Materials

Affinity gel (Affi-Gel 10), iodoacetamide (IAA) and dithiothreitol (DTT) were purchased from Bio-Rad (Hercules, CA, USA); Sequencing grade-modified trypsin (Trypsin Gold) was purchased from Promega (Madison, WI, USA). TRIZMA[®] PRE-SET CRYSTALS [tris(hydroxymethyl)aminomethane], 50 mM, pH 9.0] as a coating buffer, phosphate buffered saline with Tween 20 [PBST; 0.01 M phosphate buffer with 138 mM NaCl, 2.7 mM KCl, 0.05% (w/v) Tween 20, pH 7.4], phosphate-citrate buffer tablets (50 mM phosphate-citrate buffer, pH 5.0, 1 tablet/100 mL), hydrogen peroxide, 3,3',5,5'-tetramethyl-benzidine (TMB), BSA, *N,N'*-carbonyldiimidazole (CDI), Tween 20, horseradish peroxidase (HRP) conjugate of goat anti-rabbit IgG, sinapic acid, and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade. A Milli-Q system (Millipore, Bedford, MA) equipped with a 0.2 µm filter was used to prepare deionized water for all mobile phases. Amicon Ultra-4 centrifugal filters having 3 K regenerated cellulose membranes were obtained from Millipore (Carrigtwohill, Ireland). Chicken serum was obtained from 10 weeks old commercial broiler chickens. Chickens were fed on basal diet, which was un-medicated diet formulated to fulfill the nutritional requirements. A T-Gel absorbent column (T-Gel Purification kit, Pierce Co., Rockford, IL, USA) was used to purify polyclonal antibody (pAb). Micro Reaction vessels (2 mL with cap & septa, amber) obtained from Supelco analytical (Bellefonte, PA, USA). Microtiter plates, Maxisorp from Nunc Co. (Roskilde, Denmark) and a

microplate reader, THERMOmax from Molecular Devices Co. (Sunnyvale, CA, USA) were used for colorimetric assays.

5.3.2 Instrumentation

All proton and carbon NMR spectra were acquired on a Bruker Avance 500 MHz NMR spectrometer (Bruker BioSpin, Germany) applying a 5 mm TXI ($^1\text{H}/^{13}\text{C}$) triple-resonance inverse probe, equipped with a Z-gradient coil, and analyzed using Bruker XWIN-NMR 3.5 software. Well-resolved sharp spectral signals were obtained from NMR acquisitions at 320 K. Fourier-transform infrared (FTIR) spectra were recorded using a FTS-40 Bio-Rad spectrometer (Digilab/BioRad, Cambridge, MA, USA) operating in diffuse reflectance mode on samples dispersed in KBr (DRIFT); only diagnostic and/or intense peaks are reported. The spectra were analyzed with Bio-Rad Win-IR software (Version 4.14). Fluorescence measurements were performed on a π^* -180 PiStar stopped-flow apparatus (Applied Photophysics, Surrey, U.K.), both of the slit widths were set at 4 nm. The relative quantum yield of the standard coumarin 120 (7-amino-4-methyl-1, 2-benzopyrone) in ethanol (0.98 in EtOH) was used to calculate compound quantum yield. Absorbance of all solutions between 200 and 700 nm was below 0.7 AU. Fluorescence spectra details: - Intensity- 3 sec, Scan time- 1, Boxcar width- 0, Range- 200–1000 nm. All the solutions were excited at 354 nm.

HPLC-diode array detector (DAD) analysis was performed on an Agilent 1200 series HPLC system (Agilent Technologies Canada Inc., Mississauga, ON, Canada) equipped with a quaternary pump, an autosampler, degasser, and DAD (1024-element photodiode array, 190–300 nm). The peaks were detected at wavelengths of 214 nm with a 10 nm bandwidth corrected against a reference signal at 300 nm. Chromatography was conducted according to Olivia et al. using a Chromolith[®] SpeedRod RP-18e column (2 μm macropore size, 50 \times 4.6 mm i.d.) equipped with an online filter. The mobile phase consisted of H₂O-acetonitrile (70:30 to 30:70 in 4 min, 10:90 in 0.5 min, 70:30 in 0.5 min and equilibrated to 70:30 for 1 min) at a flow rate of 2 mL/min (Olivia, et al., 2012). A Chromolith[®] SemiPrep RP-18e column (2 μm particle size, 100 \times 10 mm i.d.) equipped with an online filter was used for preparative liquid chromatographic separations of the CLs. The mobile phase consisted of H₂O-acetonitrile (80:20 for 0.5 min and to 10:90 in 4.5 min, 80:20 in 0.25 min and equilibrated to 80:20 for 0.75 min) at a flow rate of 5 mL/min.

MS/MS of CLs was performed using a MicroTOF-Q II Mass Spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an APCI source (Parameters- dry temp- 200 °C; vaporizer temp- 450 °C; drying gas- 8 L/min; nebulizer 1.6 bar; capillary voltage- 4000 V).

MALDI-TOF-Mass spectra were acquired by matrix-assisted laser desorption ionization–time-of-flight MS using a 4800 MALDI TOF-TOF Proteomics Analyzer (Applied Biosystems, LLC, Frederick, MD, USA) equipped with a diode-pumped 355 nm Nd:YAG laser, and a laser intensity of 5500. The ion extraction delay time was set to 2000 ns and positive ion linear mode was used. Protein mass spectra were recorded as sums of 1000 laser shots. For MALDI matrix deposition, 10 mg/mL of sinapic acid was prepared in acetonitrile/water/TFA (50:50:0.1, v/v/v). The matrix (0.8 µL) was deposited on the 384-well plate (Opti-TOF insert, Applied Biosystems), followed by the application of 1.0 µL of the protein sample, then a 0.5 µL of matrix solution was spotted on top of the protein solution. After allowing the plate to dry under ambient conditions, the plate was loaded into the MS for data acquisition.

LC-ESI-QTOF-High resolution HPLC-electrospray ionization (ESI)-MS analyses were performed on an Agilent HPLC 1200 series directly connected to a MicroTOF-Q II Mass Spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an ESI source operating in positive ion mode. Protein conjugate samples were introduced onto the ESI-MS by HPLC employing a Poros R1 20 column (4.6 × 100 mm, Applied Biosystems, 20 µm) using the following linear gradient: 90:10, 0.1% formic acid/water to 10% formic acid/acetonitrile over 25 min at a flow of 1 mL/min. Ionization conditions of ESI analysis were as follows: dry gas temperature 220 °C, drying gas flow 10 L/min; nebulizer pressure 6 bar, capillary temperature 4500 V, scan range 50–20000 *m/z*. Molecular weights of the conjugates were determined from the high *m/z* scans after converting the complex spectra of multicharged protein species to the mass domain using Bruker’s deconvolution software (Compass data analysis).

5.3.3 Preparations of Affinity CLs 30 and 32

CL **30** was prepared using the procedure as described by Hering et al, (Hering, et al., 2005). CL **23** (0.16 g, 0.14 mmol) was dissolved in MeOH (5 mL) and triethylamine (0.5 mL) was added to the solution. A slurry of Affi-Gel 10 resin in MeOH (20 mL total volume with 8.30 mL settled resin volume, 0.125 mmol,) added to above solution and agitated on a shaker at 4 °C for 16 h. The gel was filtered and washed with cold MeOH and cold water sequentially, and the

combined filtrates were concentrated. The gel was added to a solution of ethanolamine (6.25 mL, 0.1 M solution in water, 0.625 mmol) in cold water (25 mL) and agitated on a shaker at 4 °C for 4 h. The gel was then filtered and washed with cold water (6 column volumes) and cold 0.2% aqueous NaN₃ (3 column volumes) and stored at 0 °C in a solution of 0.2% aqueous NaN₃ to form coupled gel **30**.

Similarly, CL **32** was synthesized from **27** using the above procedure. **27** (0.16 g, 0.15 mmol) was dissolved in MeOH (5 mL) and triethylamine (0.5 mL) was added to the solution.

Affi gel coupled CL **30** was slurry packed into a preparative column (POROS[®] PI 20 µm column (4.6 × 100 mm, 1.7 mL), Applied Biosystems[®]) and connected to Agilent 1200 series HPLC system equipped with a quaternary pump, an autosampler, degasser and diode array detector (1024-element photodiode array, 190–300 nm). The peaks were detected at wavelengths of 280 nm with a 10 nm bandwidth and against a reference signal at 360 nm. The column was first incubated with serum for 3 min and then eluted with a buffer of 0.02 M Tris-HCl pH-7.5 (Solvent A) to remove non-bound protein for 12 min and finally, eluted with (1 mg/mL of **11** in 35% MeOH/0.02 M Tris-HCl pH-7.5, Solvent B) to remove bound proteins for 15 min and the column is again equilibrated for 10 min with solvent A at a flow rate of 0.5 mL/min. The fractions were concentrated by ultrafiltration in an Amicon Ultra-4 centrifugal filter with a 3K-regenerated cellulose membrane and finally, concentrated sample was centrifuged at 10000 × g for 3 min and the resulting clear retentate solution was used for SDS-PAGE.

5.3.4 SDS-PAGE

Presence of protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Bollag, et al., 1996) using a Mini protean II system (Bio-Rad, Richmond, CA). Protein concentration was determined using a Bio-Rad protein assay dye reagent (protein solution was added to acidic dye and absorbance was measured at 595 nm, Bio-Rad, Richmond, CA). A standard curve was prepared with a BSA solution. Protein concentrations of 20–30 µg were used during gel electrophoresis. The 8% stacking gel buffer (5.28 mL sterile water; 2.03 mL 40% acrylamide; 2.5 mL 0.5 M Tris-HCl pH 6.8; 100 µL 10% SDS; 50 µL 10% APS; 8 µL TEMED) and 5% resolving gel buffer (3.01 mL sterile water; 0.64 mL 40% acrylamide; 1.25 mL 1.5 M Tris-HCl pH 8.8; 50 µL 10% SDS; 100 µL 10% APS; 8 µL TEMED) were used. The samples were diluted with sample buffer (0.6 mL

pH 6.8; 1 M Tris-HCl; 2 mL 10% (w/v), SDS; 5 mL 50% (w/v) glycerol; 0.8 mL, bromophenol blue), boiled at 95 °C for 5 min, and then applied onto a polyacrylamide gel. Electrophoresis was conducted for 75 min at a constant voltage of 100 V and then the gels were stained with Coomassie brilliant blue for 1 h. Subsequently, gels were destained using 10% acetic acid in MeOH: H₂O (40:60) and the gel image was scanned using a scanner.

5.3.5 Trypsin Digest

Proteins were digested in-gel with the MassPrep II Proteomics Workstation (Micromass, UK) following the previously described procedure (Sheoran, et al., 2005). Briefly, Coomassie-blue-stained protein gel bands are cut into pieces of ~1 mm³ and placed into 96-well plates. Protein bands were destained twice (10 minute incubations) with 100 µL of 1:1(v/v) ammonium bicarbonate: acetonitrile. Protein reduction was performed with the addition of 10 mM DTT prepared in 0.1 M ammonium bicarbonate, for 30 min at 37 °C. This step was followed by alkylation, achieved by adding 50 µL of 55 mM IAA prepared in 0.1M ammonium bicarbonate. The alkylation reaction was carried out for 20 minutes also at 37 °C. Gels are washed and dehydrated before being saturated with 25 µL of 6 ng/µL trypsin prepared in 50 mM ammonium bicarbonate; digestion was carried out at 37 °C for 5 h. CLs were extracted with 30 µL 0.1% trifluoroacetic acid/3% acetonitrile for 30 min, and then twice with 24 µL, 0.1% trifluoroacetic acid/50% acetonitrile for 30 min. The combined extracts were dried in a SpeedVac System (Model DNA 120, Thermo Savant DNA SpeedVac System, San Diego, CA). Samples were reconstituted in 40 µL of 0.1% trifluoroacetic acid/3.0% acetonitrile for LC-ESI MS analysis (or in 10 µL of the same solvent for MALDI TOF/TOF analysis).

5.3.6 Production of Antisera

Preparation of Immunogen (**33** and **34**)

The immunogen was prepared by conjugating CLs (**24**, MW 1177.6 Da; **28**, MW 1080.6 Da) with BSA (MW 66462.2 Da), according to the method of Lee et al. (Lee, et al., 2000). **33** conjugate was prepared as follows: **24** (10 mg, 8.5×10^{-3} mM) and CDI (5.4 mg, 3.3×10^{-2} mM) were added to a 2-mL micro reaction vessel containing a triangular stir bar. Tetrahydrofuran (THF, 0.6 mL) was added and the reaction mixture stirred continuously at room temperature and 0.15 mL (×4) of reaction mixture was added slowly to a continuously stirred solution of BSA (2.0 mg, 3.0×10^{-5} mM) dissolved in 1 mM sodium borate (1 mL, pH 8.5) for different time

intervals (4, 12, 16, and 24 h). The reaction was stirred for 48 h at room temperature and the solutions were then filtered and centrifuged ($\times 6$) against a 30000-MW cutoff filter with deionized distilled water (50 mL), until pH was neutral. The filtrate was then rinsed from the filter, diluted to a concentration of 1 mg/mL in deionized distilled water, and stored at $-20\text{ }^{\circ}\text{C}$. The **34** conjugate was also obtained using the method described above, except that the **28** (10 mg, 9.2×10^{-3} mM) was used instead of the **24** (10 mg, 8.5×10^{-3} mM).

Rabbits were immunized with emulsions prepared from conjugates of **33** and **34** at the Animal Care Unit, College of Veterinary Medicine using approved animal care protocols (Animal and Ethics Committee, University of Saskatchewan, Saskatoon, SK, Canada). First, immunogen concentration was 1.0 mg protein/mL in 10 mM PBS containing 138 mM NaCl and 2.7 mM KCl at pH 7.4. An aliquot of the protein solution, containing approximately 150 μg of protein, was then mixed with TiterMax[®] Gold (CytRx, Norcross, GA, USA) in a 1:1 ratio (v/v). The selected animal (~ 3 kg) received the prepared immunogen through subcutaneous administration. Test bleeds were taken 3 weeks after the 1st immunization and 1 week after each boost. Immunization boost was given after third week and continued until fifth week. The antisera titre was determined by a non-competitive indirect enzyme linked immunosorbent assay (NCI-ELISA) as described in section 5.2.8. Blood (80 mL/animal) was collected 1-week after the last booster injection, which gave the highest antiserum titre. The antisera were isolated from the blood by centrifuging at 3000 g for 20 min with NaN_3 (0.02%, w/v), and stored at $-70\text{ }^{\circ}\text{C}$ until use.

5.3.7 Purification of Polyclonal Antibodies

The purification of immunoglobulin G (IgG) type antibodies was performed using a T-Gel purification kit and gel filtration chromatography (Sephadex G-25). Briefly, antisera (1 mL) was mixed with K_2SO_4 to a 0.5 M solution, centrifuged at 10000 g for 20 min, and then filtered through a 0.45- μm syringe filter (Polytetrafluoroethylene membrane, Sartorius Co., Göttingen, Germany). The filtrate was loaded onto a T-Gel column (thiophilic adsorption, column contains a sulfone group in proximity to thioether group which helps in binding with proteins) equilibrated with a pH 8.0 binding buffer (provided by the manufacturer) containing 0.5 M potassium sulfate and then washed with 50 mM sodium phosphate buffer at pH 8.0. The eluted IgG protein fractions (280 nm absorbing fractions) were pooled. Fractions (1.5 mL) were

desalted using a Sephadex G-25 column (15 × 170 mm) then equilibrated with PBS. UV absorbance (280 nm) was determined for each fraction. The fraction showing the highest absorbance was assumed to contain IgG Ab. IgG concentration of 8 mg protein/mL was recovered from 10 mL of antiserum through this purification.

5.3.8 ELISA Protocol

In the NCI-ELISA with **33**, each well of polystyrene microtiter plate was coated with 100 µL of **33** conjugate (2 µg/mL) in a coating buffer (0.05 M Tris, 0.15 M NaCl buffer, pH 9.0). Antigen-treated plates were stored overnight at 4 °C, and each well was washed three times with buffer (150 µL, 0.02 M Tris, 0.15 M NaCl buffer, pH 7.4, with 0.02% Tween 20). The anti-**33** antiserum (100 µL) diluted with the PBS buffer (dilution 1/10000, v/v) containing 1% BSA as a blocking reagent, was added to each well and left for 1 h. After washing three times, goat anti-rabbit IgG-HRP conjugate (100 µL), as secondary Abs diluted to 1/5000, 1/7000, and 1/10000 (v/v) in the PBS buffer, was added to each well and left for 1 h. After washing three times, substrate solution (100 µL, 0.01% TMB, 0.05 M phosphate citrate buffer, pH 5.0, 0.002% H₂O₂, v/v) was added, developed for 30 min at room temperature, and the reaction was stopped with 2 M H₂SO₄ (50 µL). Absorbance was measured at 450 nm with a microplate reader. The protocol for the NCI-ELISA coating with CL antigen was similar to that previously described (Otvos & Szendrei, 1997). Briefly, 50 µL of **24** or **28** (10 µg/mL in 80% isopropyl alcohol/water) was added to wells of a microtiter plate. The plate was kept at 37 °C for 2 h to dry the antigen on the plate. After washing 3 times with PBST, 100 µL of 1% BSA-PBS as a blocking solution was added and allowed to incubate at 37 °C for 1 h. The plate was washed as described above, and 50 µL of diluted anti-**33** antiserum or anti-**34** antiserum was added. After incubation at 37 °C for 1 h and washing the plate, 100 µL of goat anti-rabbit IgG-HRP conjugates (1/3000 dilution in 1% BSA-PBS) was added. The plate was incubated at 37 °C for 1 h and then washed. After washing three times, the coloring reaction was the same as described for the NCI-ELISA.

In the Competitive Indirect Enzyme Linked Immunosorbent assay (CI-ELISA), the procedure was the same as in the NCI-ELISA, except that 100 µL of **24** solution and anti-**33** antiserum, diluted to 1/10000 in the washing buffer containing 1% BSA (1:1 mixture), were added to each well of the **33** conjugate-coated plate in place of the antiserum used in the experiment of the NCI-ELISA.

5.4 Results and Discussion

In this study, we have selected two types of CLs, **11** and **10** containing nine and eight amino acid residues. However, **11** and **10** contain Msn moieties and are oxidised products of CLs (**2** and **3**) and CLs (**13** and **5**) with the same basic core. We have already devised a method to systematically modify the Met in the parent CLs (**2** and **13**) without changing other amino acids (Chapter 4). CLs were produced that contain reactive -NH_2 and -OH groups that can be used to selectively couple intact CLs with other reactive functional groups present in fluorescent compounds, affinity media and protein (BSA) designed for binding these reactive groups. In this study, amino analogs of **10** and **11** were reacted with a succinimidyl ester of coumarin dye and affinity gel to form a covalent bond. We have developed a general synthetic protocol and could be used to prepare various fluorescent CL derivatives from commercially available dyes with reactive groups. The fluorescence spectra of these compounds were obtained in different polarity of solvents to understand the photophysical behaviour of CL/coumarin complexes. The hydrophobic surfaces and cyclic conformational structure makes these CLs good candidates to bind proteins. CLs consist of two different surfaces, hydrophobic surface formed by aromatic side chains and the other hydrophilic, formed by CL backbone that helps in binding with protein. The analogue of **1** has shown binding to proteins from cytosol and plasma membranes of rat hepatocytes (Kemmer, et al., 1997). However, there was no study on the binding of chicken serum proteins with CLs. Hence CL affinity matrix was used to study the binding of chicken serum proteins with CLs. In addition, alcohol analogs (**24** and **28**) were coupled with the lysine of BSA through CDI activation so that the CLs might act as hapten to induce antibody production when injected into rabbits. pAbs recovered from the rabbit serum can be used in two ways: for localization of CLs when they are introduced to a medium or biological material or detection of CLs in flax and flaxseed products. The total concentration of CLs is only 0.2% of flaxseed and various groups have isolated CLs (Brühl, et al., 2007; Matsumoto, et al., 2001; Morita, et al., 1999; Morita, et al., 1997). NMR and HPLC-MS were utilized to identify and characterize the CLs. However, they are not satisfactory for the detection of CLs in multi-component mixtures such as oil, fat, wax and grease. In contrast, an enzyme-linked immunosorbent assay (ELISA) provides highly sensitive, rapid, and precise means to analyze a large number of samples. ELISA uses enzyme and antibody. Antigen quantification of nanograms is possible. **11** and **10** are the oxidized products of their parent CLs **3** and **5** and are

not present in freshly ground flaxseed but may be indicators of ageing and oxidation flaxseed (Jadhav, et al., 2013) (Chapter 3).

5.4.1 Synthesis of CL Conjugates

The analogs of CLs (**11** and **10**) containing amine (**23** and **27**) and hydroxyl side chain (**24** and **28**) were synthesized previously (Chapter 4). Amine reactive groups of **23** and **27** were coupled with coumarin succinimidyl ester dye to form fluorescent derivatives **29** and **31** respectively. Similarly **23** and **27** were also coupled with the succinimidyl ester of affinity matrix to form CL-matrix **30** and **32**, respectively (Figure 5.1). These reactions form a stable covalent amide bond between CL and dye, matrix with a loss of *N*-hydroxysuccinimide. Alcohol reactive groups of **24** and **28** were used to couple with the BSA. Initially, hydroxyl group of CLs (**24** and **28**) was activated with CDI and then reacted with the lysine of BSA to produce CL-BSA conjugates **33** and **34**, respectively (Figure 5.2).

5.4.2 Characterization of CL Conjugates

5.4.2.1 Characterization of Fluorescent Derivatives **29** and **31**

The protons attached to the heteroatoms were assigned from ^1H -NMR, and their coupling to amide protons and carbonyl carbons employed NOE and HMBC correlations (Figure 5.3). These elucidated the amino acid sequence of the CL. ^1H - ^1H COSY was used for assignment of β , γ and δ protons while elucidation of the attachments of these protons to carbon atoms was determined by HMQC. The MS/MS and NMR spectral data of **29** (Figure 5.4 and experimental section) showed similarities to that of **11** (Jadhav, et al., 2013). However, a fragment loss of m/z 451 in the MS/MS data of **29** (Figure 5.4) in place of the loss of m/z 163 (Msn in **11**) suggested the presence of an attachment of coumarin molecule to Msn₁ residue in **29**. The amino acid sequence of **29** was verified using tandem MS/MS fragmentation (Figure 5.4). MS/MS profile of **31** reveals the amino acid sequence (Figure 5.5). However, the ^{13}C NMR spectrum showed two sets of signals for each carbon atom corresponding to two CL conformers of **31** similar to **10** (Jadhav, et al., 2013). Signals from the major conformer are shown in the experimental section. The structures of **29** and **31** were confirmed by MS/MS and NMR methods. HPLC-MS analysis showed a $[\text{M}+\text{H}]^+$ ion peak at m/z 1378.6751 corresponding to a molecular formula, $\text{C}_{70}\text{H}_{96}\text{N}_{11}\text{O}_{16}\text{S}$ of **29** and m/z 1281.6317 corresponding to $\text{C}_{65}\text{H}_{89}\text{N}_{10}\text{O}_{15}\text{S}$ of **31**.

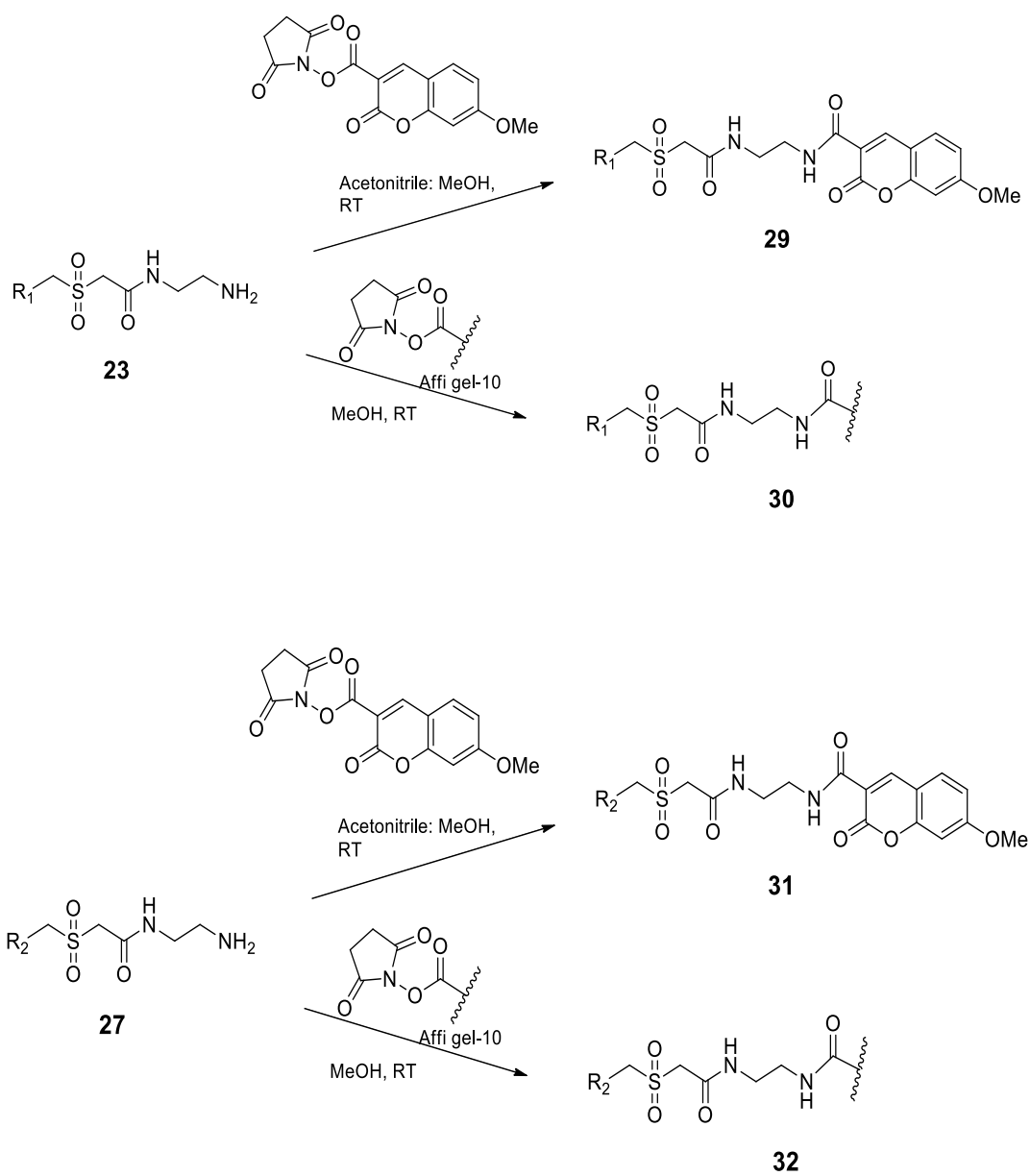


Figure 5.1 Synthesis of fluorescent derivatives (**29**, **31**) and CL affinity matrices (**30**, **32**)

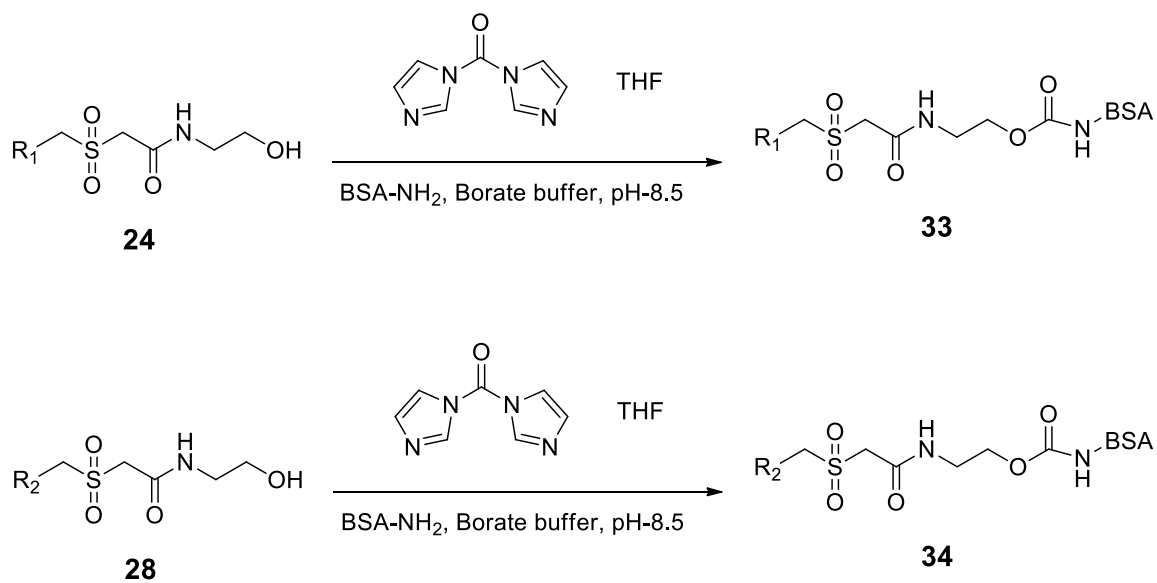


Figure 5.2 Synthesis of CL-BSA conjugates **33** and **34**

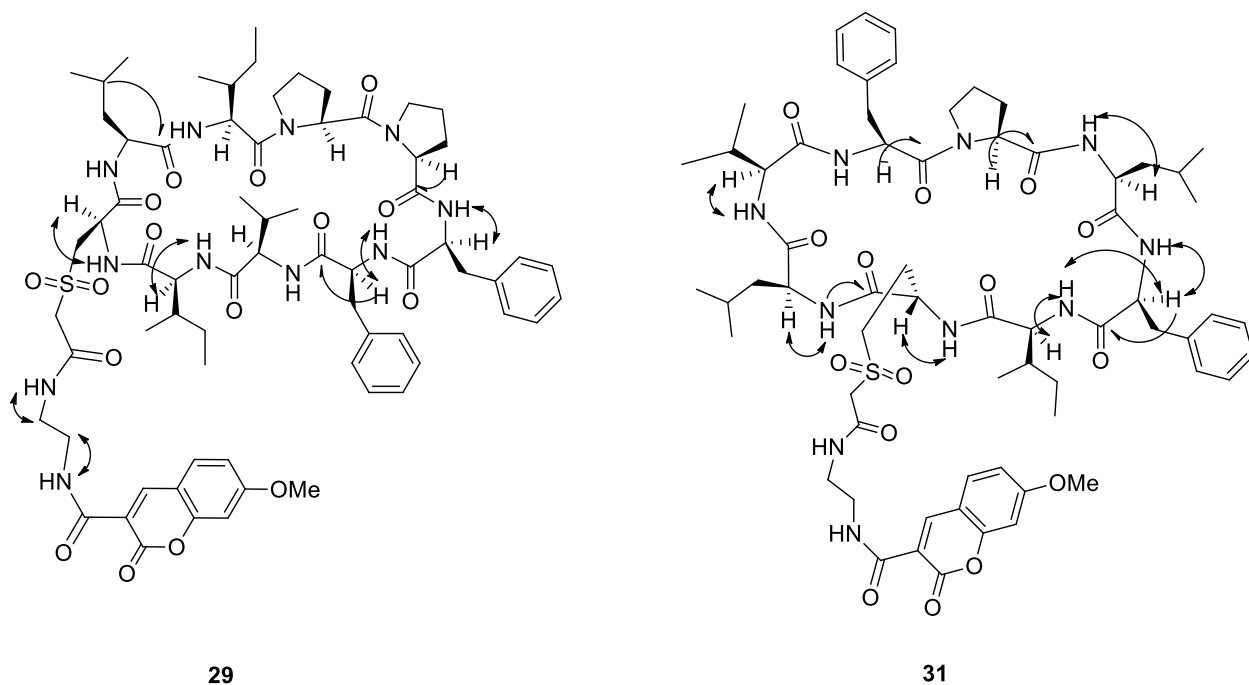


Figure 5.3 Structures of **29** and **31**. Double arrows show selected NOE correlations. Half arrows show selected HMBC correlations

Following the preparation of fluorescent CLs **29** and **31** it was important to investigate the occurrence of structural changes in the CL core structure. NMR spectroscopy was therefore used as a tool to compare the structural signals of the starting material **11** and **10** to those of the conjugate products **29** and **31**, respectively. The α protons of each amino acid residue were used to determine if measurable structural changes took place (Dekan, et al., 2011). Our observation from chemical shift analyses showed that most of the amino acid residues (six out of nine and five out of eight amino acid residues for **29** and **31** respectively) shows no significant perturbations in the NMR signals of **29** and **31** compared to mother compounds **11** and **10**, respectively (Table 5.1). We therefore concluded that the CLs **29** and **31** might have structural similarity to their parent CLs.

5.4.2.2 Characterization (Coupling efficiency) of Bound Ligand and Matrix

The coupling efficiency of stationary phase containing coupled modified CLs and matrix can be determined by performing elemental analysis (C, N and S) on the blank gel and CL-coupled matrix **30** and **32** (Table 5.2). In this analysis, samples were first fully combusted and then the products of its combustion such as carbon dioxide, water, nitric oxide and sulfur dioxide were analyzed. The difference in % of S between blank and modified gel was used to determine the coupling efficiency. The analysis shows almost 70% coupling of CLs to the gel in **30** and about 38% in **32**.

5.4.2.3 Characterization of BSA Conjugate

The CL-BSA conjugates (**33** and **34**) were characterized using SDS-PAGE, MALDI and ESI-QTOF. These analyses indicate binding of haptens (CLs) to BSA.

SDS-PAGE. This technique determines protein molecular weight. Incorporation of haptens covalently bound to the protein would increase their molecular mass and possibly decrease their mobility in the SDS gel. After conjugation, bands attributed to the conjugate of CL with BSA were broader and less mobile than unreacted BSA (66 kDa) (Figure 5.7). This result is consistent with the formation of BSA covalently bound to CL. However, The CL-BSA conjugates have shown smeared bands in gel electrophoresis. This prevents the determination of the exact molecular mass (es) of the CL-BSA conjugates as compare to BSA (Adamczyk, et al., 1994). As SDS-PAGE has lower sensitivity in mass discrimination (<5%) than MS techniques, it can't determine exact number of haptens incorporated into the protein (Barinaga, 1989).

MALDI. MALDI has been used to determine the molecular weights of proteins (Figure 5.7) (Adamczyk, et al., 1994). The average number of haptens incorporated into immunogen was determined from the difference in the measured molecular weights of the conjugate from non-modified BSA. The reported molecular mass of BSA is 66430.3 Da (Hirayama, et al., 1990) and CLs **24** and **28** were 1176.6 Da and 1079.6 Da with loss of hydrogen during conjugation, respectively. However **33** conjugate shows molecular masses of 44587.7 ($M + 2H$)²⁺ = 89175.4 Da and 77761.2 Da implying incorporation of haptens. **34** conjugate also shows hapten addition with a molecular mass of 44928.4 ($M + 2H$)²⁺ = 89856.8 Da, 78918.0 Da and 68074.5 Da.

LC-ESI-QTOF Mass Spectrometry. Molecular weights of **33** and **34** conjugates were recorded after deconvolution of mass spectra. Addition of one molecule of **24** to BSA increased molecular mass of the CL-BSA conjugate by 1204.6 Da [$1176.6 + 28$ (CO)] while addition of **28** would increase mass by 1107.6 Da [$1079.6 + 28$ (CO)]. A molar mass of 93591.9 for **33** conjugate ($93591.9 - 66430.3 = 27161.6$), as observed by MALDI, indicates that 22.5 haptens (**24**) were attached to BSA. There were different numbers of incorporation of haptens based on various molecular masses (Table 5.3).

5.4.3 Applications of CL Conjugates

5.4.3.1 Fluorescence Studies of **29** and **31**

The absorption and fluorescence studies were conducted in four different solvents (methanol, ethanol, acetonitrile and dimethylformamide) at a concentration of 3×10^{-5} M. The samples were excited at 354 nm, and the emission spectrum was recorded. Coumarin 120 (7-amino-4-methyl-1, 2-benzopyrone) was used as reference (Bryantseva, et al., 2008). 7-methoxy coumarin in which methoxy group acts as electron donor and carbonyl as acceptor was used as tag. The fluorescence intensity of this coumarin dyes is solvent dependent and it increases with solvent polarity with negligible emission shift (Heldt, et al., 1995; Muthuramu & Ramamurthy, 1984). It has been postulated that, $n\pi^*$ and $\pi\pi^*$ singlet states are close to each other in the non-polar solvent, where intersystem crossing takes place and hence the fluorescence yield is lower. However in polar solvent the difference between these two states is larger, making intersystem crossing inefficient (Muthuramu & Ramamurthy, 1984). **29** and **31** absorb light at 347 nm and emit light at 406 nm. Both show the same trend having higher quantum yields in methanol and ethanol than in either acetonitrile or DMF (Table 5.4 and 5.5).

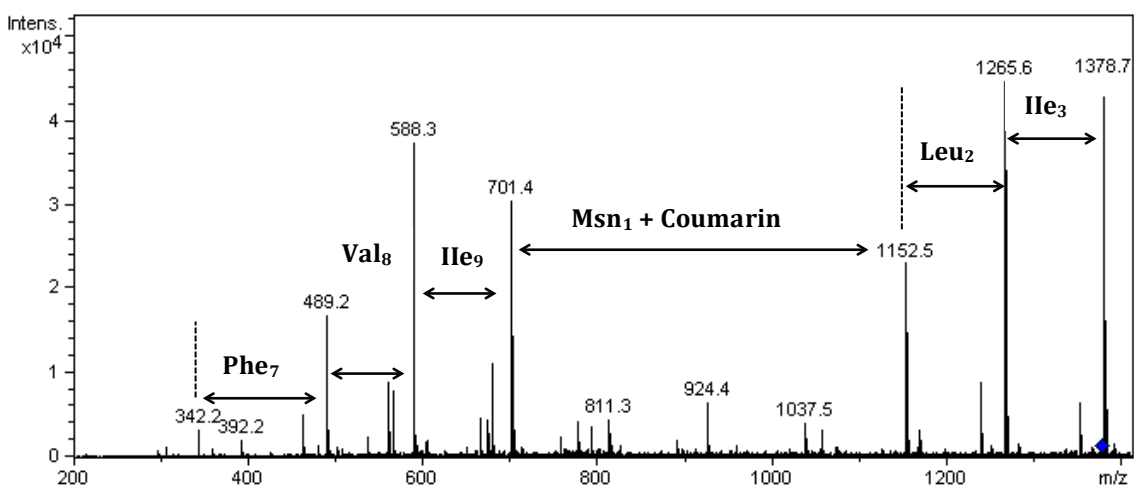


Figure 5.4 CID MS/MS spectrum of **29** showing fragmentation pattern

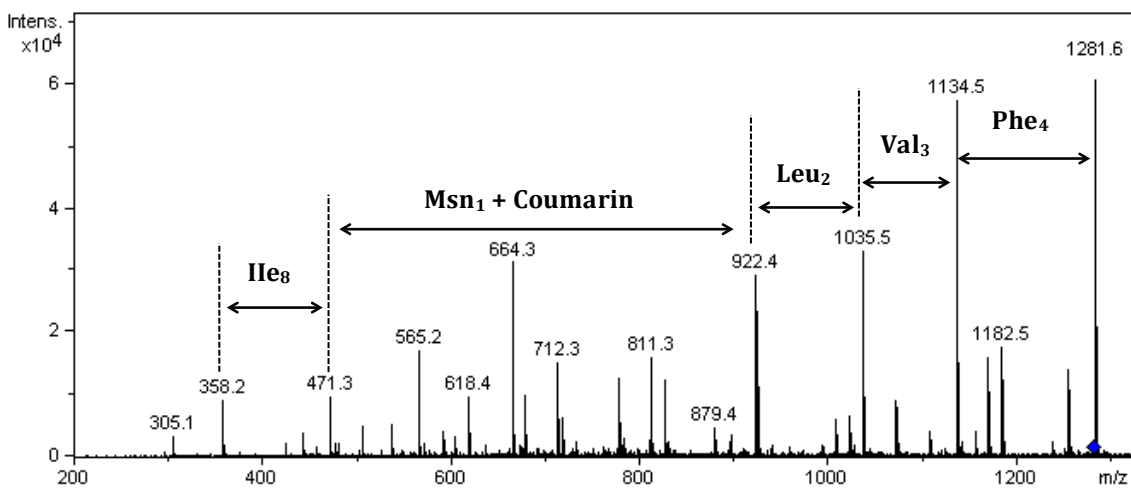


Figure 5.5 CID MS/MS spectrum of **31** showing fragmentation pattern

Table 5.1 Chemical shift differences of α H signals for **29** and **31** compared with the **11** and **10**, respectively.

α H assignment	11	29	α H assignment	10	31
Msn ¹	4.81	4.76	Msn ¹	4.76	4.63
Leu ²	4.07	3.83	Leu ²	4.36	4.33
Ile ³	4.52	4.47	Val ³	4.50	4.42
Pro ⁴	3.90	3.78	Phe ⁴	4.34	4.42
Pro ⁵	4.07	4.11	Pro ⁵	4.09	4.12
Phe ⁶	4.11	3.82	Leu ⁶	3.81	3.60
Phe ⁷	4.96	4.86	Phe ⁷	4.96	4.97
Val ⁸	3.90	3.86	Ile ⁸	4.13	3.90
Ile ⁹	4.60	4.55			

Table 5.2 Elemental analysis of blank and CL gels (**30** and **32**).

	C	N	S
Blank gel	43.10	4.692	0.000
30	46.42	4.201	0.581
32	44.68	3.879	0.293

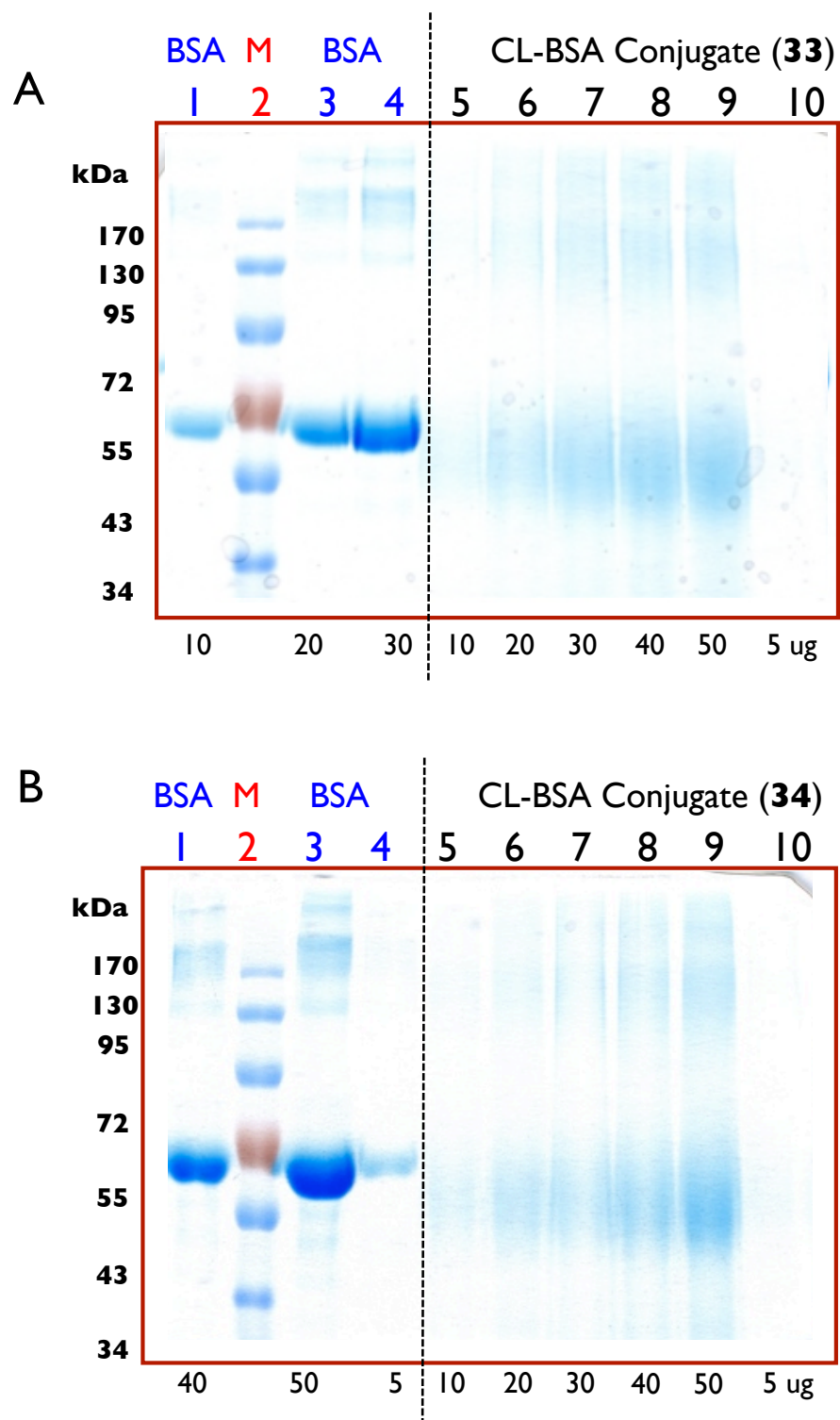


Figure 5.6 SDS-PAGE patterns of **33** (A) and **34** (B) conjugates

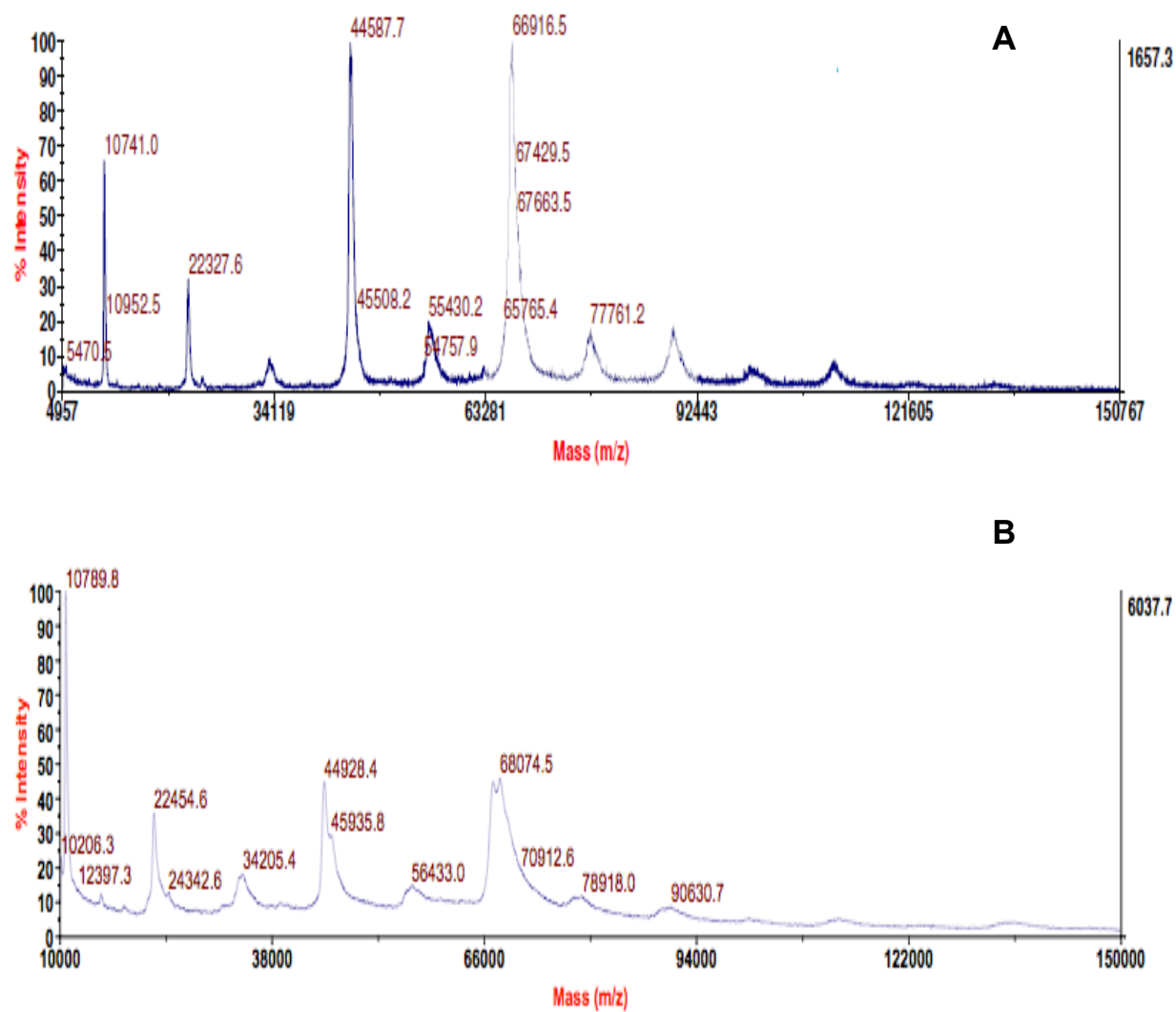


Figure 5.7 MALDI-TOF mass spectra of **33** (A) and **34** (B) conjugates

Table 5.3 Molecular masses of **33** and **34** conjugates.

33 conjugate	34 conjugate
93591.9 ($M_{BSA} + 22.5$ HAP, 100)	93612.7 ($M_{BSA} + 24.5$ HAP, 100)
81290.4 ($M_{BSA} + 12.3$ HAP, 84)	66746.4 ($M_{BSA} + 0$ HAP, 90)
84522.5 ($M_{BSA} + 15.0$ HAP, 82)	90085.1 ($M_{BSA} + 21.4$ HAP, 80)
84637.2 ($M_{BSA} + 15.1$ HAP, 81)	76398.7 ($M_{BSA} + 9.0$ HAP, 78)
84598.2 ($M_{BSA} + 15.0$ HAP, 79)	
81485.6 ($M_{BSA} + 12.5$ HAP, 71)	
81462.4 ($M_{BSA} + 12.5$ HAP, 56)	
$M_{conjugate}$ ($M_{BSA} + \text{average number of haptens, relative intensity}$)	

The hydrogen bonding of hydroxyl group of alcohols have extra effect as compare to both aprotic acetonitrile and DMF. However, fluorescence in DMF occurs with very low quantum yield as compare to fluorescence in other solvents. Potentially non-radiative decay or conformational changes occur in the dye that arises from interaction with the CL in DMF lowering the quantum yield.

Fluorescent tags have been successfully used for imaging proteins inside cells and are valuable in establishing the fate of a given protein or chemical compound in biochemical processes. (Ferro-Flores, et al., 2010). Some of the examples of fluorescent-labeled peptide include peptide-dye conjugate [arginine-glycine-aspartic acid (RGD)-Cy5.5] has shown affinity to integrin receptor as contrast agent *in vitro*, *in vivo*, and *ex vivo*. The ϵ -amino group of lysine residue present in RGDyK peptide was coupled with *N*-hydroxysuccinimide ester of cyanine 5.5 dye to use as a fluorescent probe to detect U87MG glioblastoma xenograft with high contrast (Chen, et al., 2004). The biological activity of CLs makes them good candidates to be used them as fluorescent tag. However there was no report on the attachment of fluorescent dye to CLs. In this study we are attaching a coumarin dye to CLs through a covalent linkage. NMR studies shows that the CL dye conjugate molecule might have structural similarity with the parent molecule CLs **10** and **11**. Hence they can be used for fluorescent imaging and active side chain chain to link any type of dye. The polar protic solvent have shown higher quantum yield as compare to aprotic solvent. The hydrogen bonding has extra effect and makes intersystem crossing between $n\pi^*$ and $\pi\pi^*$ singlet states inefficient.

5.4.3.2 Affinity Purification using CL Affinity Matrix **30**

Amine reacting side chain of **30** was used to couple with the succinimidyl ester of Affigel 10 matrix. This forms a stable amide bond between CL and matrix with a loss of *N*-hydroxysuccinimide. The affinity column was directly attached to an HPLC solvent delivery system and compounds eluting from the column were monitored at 280 nm. The chromatogram shows two peaks where the first peak represents the bound protein and the second unbound protein (Figure 5.8). Initially the column was eluted with Tris buffer to remove unbound protein. Subsequently, **11** was incorporated in the elution solvent to enable the removal of bound compounds. As **11** has low solubility in Tris buffer, a mixture of methanol and Tris buffer was used to dissolve this CL.

Table 5.4 Spectroscopic analysis of **29** in different solvents.

Solvent	λ_{max}	$\lambda_{\text{emission}}$	ϕ Quantum yield
Ethanol	348	407	0.69
Methanol	348	404	0.71
Acetonitrile	345	402	0.57
Dimethylformamide	347	408	0.44

Table 5.5 Spectroscopic analysis of **31** in different solvents.

Solvent	λ_{max}	$\lambda_{\text{emission}}$	ϕ Quantum yield
Ethanol	348	403	0.62
Methanol	347	403	0.68
Acetonitrile	345	405	0.59
Dimethylformamide	347	406	0.42

The column was also eluted with Tris buffer-methanol before **11** was added to confirm that methanol addition was not eluting the protein. This solvent without **11** did not elute additional protein from the column. The columns were regenerated by elution with methanol.

SDS-PAGE

Bound protein fractions released by elution with **11** were added to a SDS-PAGE electrophoresis gel. Coomassie blue shows a single band at 28 kDa (Figure 5.9 lane 4). The affinity-purified fraction (bounded protein) was compared to crude serum (Figure 5.9 lanes 2 and 3). This band was excised and the protein digested with trypsin as described in the Materials and Methods. The resulting CL was sequenced and amino acid sequences thus obtained (Table 5.6) was identical to that of a chicken apolipoprotein A1. The matched amino acids were shown in red and it shows 58% protein coverage and it consists of 246 amino acids.

Hydrophobicity of CLs plays a significant role in transport across cell membranes in addition to distribution in tissues and organs (Kemmer, et al., 1997). Hydrophobic peptides such as the somatostatin analogue 008, linear hydrophobic peptide EMD 55068 and **1** analogue CDP [c(-Ala-Lys-Pro-Phe-Phe-Ala-Lys-Pro-Phe-Phe-)] have shown binding to proteins in the plasma membranes and cytosol of rat hepatocytes. The specific binding proteins in the plasma membranes are cytochrome P450 II C13, cytochrome P450 II C22 and 3-hydroxyandrogen-UDP-glucuronosyltransferase. In the cytosol, the glutathione-S-transferase is the binding protein. **1** and its analogues have shown two surfaces, aromatic side chain forms hydrophobic and the peptide backbone forms a hydrophilic surface. These hydrophobic surfaces and cyclic conformational structure helps these compounds to bind proteins (Kemmer, et al., 1997). Hence it was interesting to study the binding of chicken serum proteins with analog **11** via affinity chromatography. Amino side chain of **23** and **27** is accessible for covalent linkage with affinity matrix. In our study we have identified a specific binding of protein Apo A1 with **23**. The main function of Apo A1 is to reverse cholesterol transport and cholesterol efflux. It was reported that CsA inversely affects the Apo A1 uptake, degradation and resecretion. If CLs binds with Apo A1, then it might act as inhibitor and may interfere in cholesterol homeostasis. This study will help to understand the effect of binding on the biological activity of CLs.

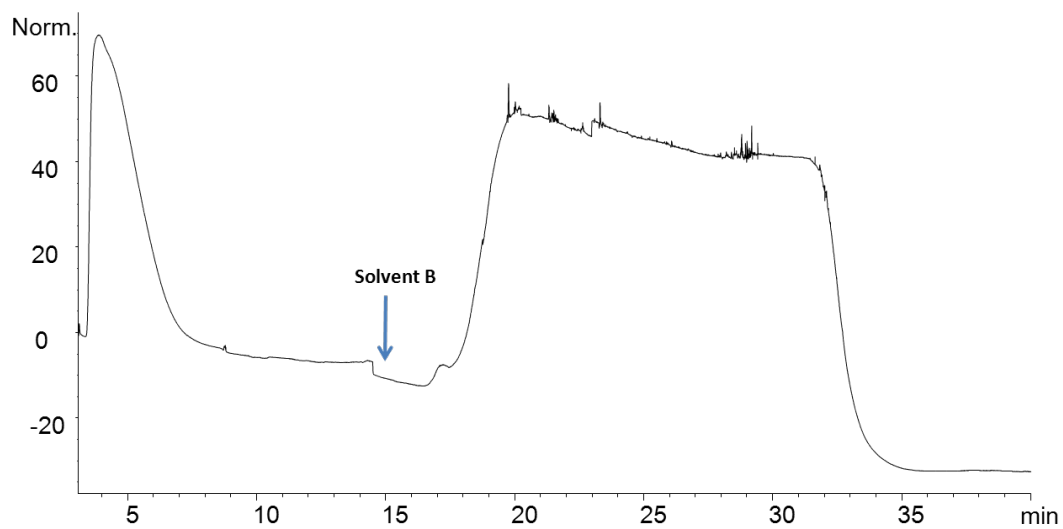


Figure 5.8 Elution profile of crude serum

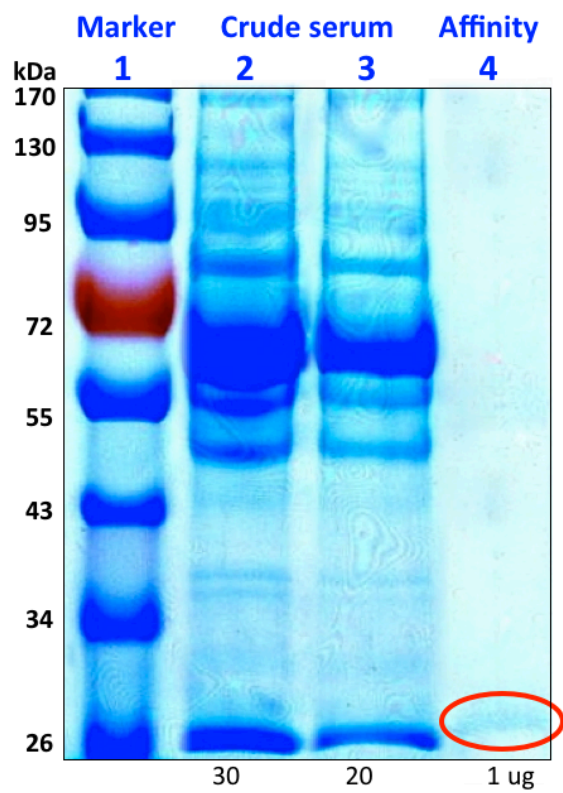


Figure 5.9 Coomassie blue stained gel-showing electrophoresis of Apo A1 purification

5.4.3.3 ELISA Study using Polyclonal Antibodies against 33 and 34

When the properties of two polyclonal antibodies (pAbs) produced in this study were compared, the recognizing pattern of the anti-**33** pAb toward antigens was similar to that of the anti-**34** pAb. In this study, the CI-ELISA was developed, and the feasible test of optimized immunoassay was performed. The standard curves showed the detection limit and the ranges of the competitive indirect method using the anti-**33/34** pAbs (Figures 5.10 and 5.11). In the CI-ELISAs (Figure 5.11), the detection limit of **28** by the anti-**34** pAb with the blocking buffer (1% BSA in PBS) was 0.01 $\mu\text{g/mL}$, whereas without the blocking buffer it was 0.1 $\mu\text{g/mL}$, indicating that with blocking buffer was 10 fold more sensitive than without the blocking buffer. However, in the CI-ELISA using the anti-**33** pAb, the detection limit of **24** was approximately 0.01 $\mu\text{g/mL}$ (ppm) and almost the same results were obtained with and without the blocking buffer.

Therapeutic peptides have gain importance as drug candidates in different applications including anti-microbial therapy, anticancer vaccines, Alzheimer's therapy, and anti-malarial (Ganz & Lehrer, 1998; Ghosh, et al., 2001; Soto, et al., 1998; Zwaveling, et al., 2002). Hence it is important to monitor the stability of the drug inside the body and antibodies play an important role (Ohara, et al., 2011). Some of the examples include cyclic hydrophobic peptides such as immunosuppressant cyclosporine A (CsA) and growth inhibiting hormone somatostatin have been used to produce polyclonal antibodies. The pAbs of CsA were used to monitor the cyclosporine levels using radioimmunoassay and somatostatin pAbs were used to detect the presence of somatostatin receptor in carcinoid tumors (Cacalano, et al., 1989; Janson, et al., 1998). However there were no reports on the generation of pAbs against flax CLs. In this study CLs were conjugated to BSA and the CL-BSA conjugates were used to generate pAbs in rabbits. Hence pAbs generated against CLs can be used to develop competitive indirect ELISA (CI-ELISA) for the detection of CLs in complex matrices. It was reported that CL **5** is responsible for the bitter flavor of flaxseed oil (Brühl, et al., 2007). Since these generated pAbs will have affinity to CL **5** and might be able to detect CL **5** (bitter flavor) in oil. They can be use for both therapeutic drug monitoring and detection in flaxseed samples. Affinity between molecules and other molecules or solids depends on three dimensional structures (Segura, et al., 2007). In this study the effect of modifying CLs with an activating group has the potential to change the backbone conformation of CL molecule and thus change the affinity with other molecule.

Table 5.6 Apo A1 peptide sequence.

1	RSFWQHDEPQ	TPLDRIRDMV	DVYLETVKAS	GKDAIAQFES	SAVGKQLDLK
51	LADNLDTLA	AAAKLREDMA	PYYKEVREMW	LKDTEALRAE	LTKDLEEVKE
101	KIRPFLDQFS	AKWTEELEQY	RQRLTPVAQE	LKELTKQKVE	LMQAKLTPVA
151	EEARDRLRGH	VEELRKNLAP	YSDELRQKLS	QKLEEIREKG	IPQASEYQAK
201	VMEQLSNIRE	KMTPLVQEFR	ERLTPYAENL	KNRLISFLDE	LQKSVA

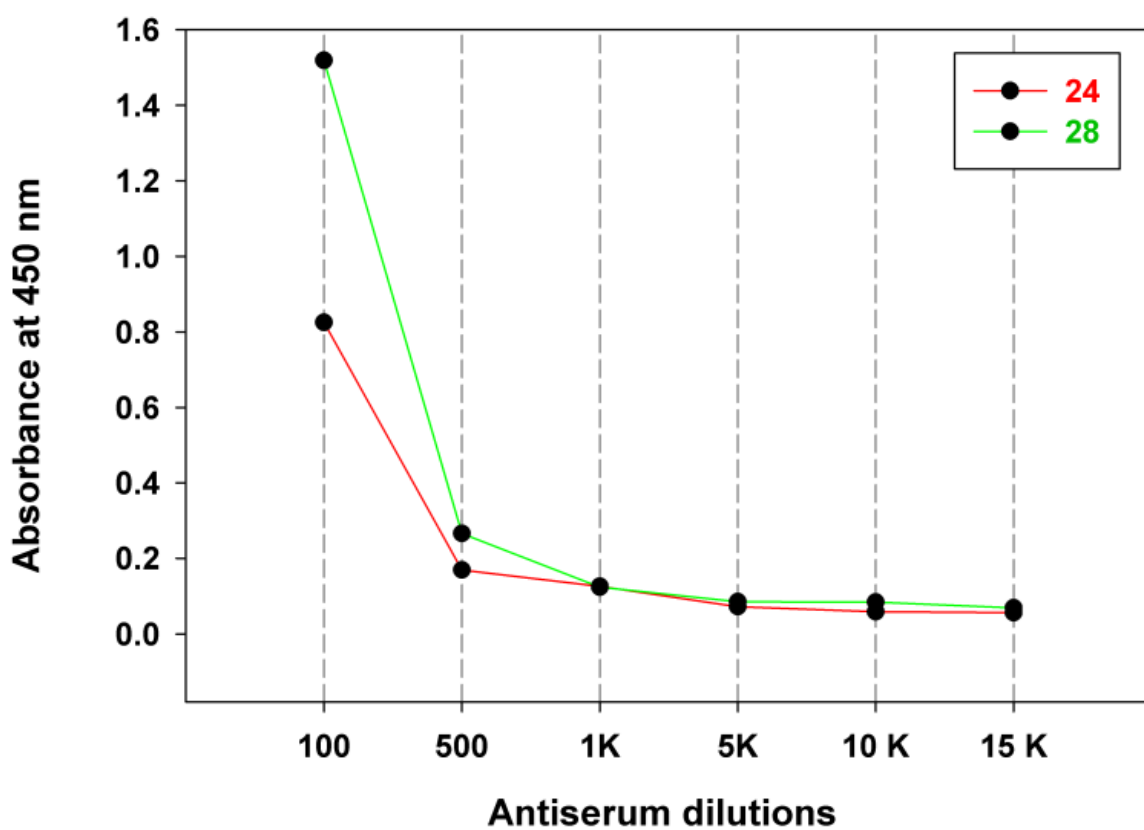


Figure 5.10 Binding of 10 μ g of 24 or 28 to anti-33 pAb or anti-34 pAb

CLs covalently attached to coumarin dye, affinity matrix and BSA through a sulfone side chain may not have the same conformation as the native unmodified CL. For example, glycopeptides binding to major histocompatibility complex (MHC) can elicit a T-cell response if the glycan moiety is attached at suitable position to the peptide. The substitution of the linkage of the glycosyl peptide with serine for threonine did not affect binding. Also in this model system there is no effect of configuration of the glycan moiety (α or β linkage). Thus the atoms involved in the covalent linkage between glycan and peptide were hypothesized to be inaccessible to MHC and have negligible effect on binding affinity (Galli-Stampino, et al., 1997). These studies have shown that if the glycan is situated outside the MHC affinity region of the peptide, it may not be recognized (Harding, et al., 1993). This effect may be caused by an indirect effect where the glycan changes the conformation of the peptide. However if glycan is situated at the region of the molecule involved in affinity it will typically affect binding (Ishioka, et al., 1992). If the glycan protrudes away from binding site then the binding is typically maintained (Deck, et al., 1995). CL **1** has shown immunosuppressive activity by complexing with cyclophilin and it was reported that sequence of the binding region is Val-Pro-Pro-Phe (Gallo, et al., 1995). However, this binding region is opposite to the sulfone side chain and, thus, may not be affected by covalent attachment of the linking moiety. The conformation of the peptide backbone can be determined by studying the ^1H -NMR shift of the alpha protons of amino acids. Therefore, the peptide backbone of parent and modified CLs were compared to determine if modification led to structural changes. Here we report that the chemical shifts of alpha proton for most of the amino acid residues of parent and modified CLs were similar and hence the conformational structure may have been intact. However to confirm the affinity fragment responsible for binding, chemical modification at the binding region should be performed.

5.5 Experimental Procedures

Fluorescent Derivatives (**29** and **31**)

A mixture of CL **23** (0.2 g, 0.05 mmol) and 7-methoxycoumarin-3-carboxylic succinimidyl ester (1.5 eq, 0.08 g) was dissolved in a solution of 30% methanol/acetonitrile and triethylamine (1 eq, 0.02 g) was added. The reaction mixture was stirred at room temperature for 2h. The organic solvents were evaporated and crude product was purified using preparative-HPLC to obtain **29** in 0.24 g.

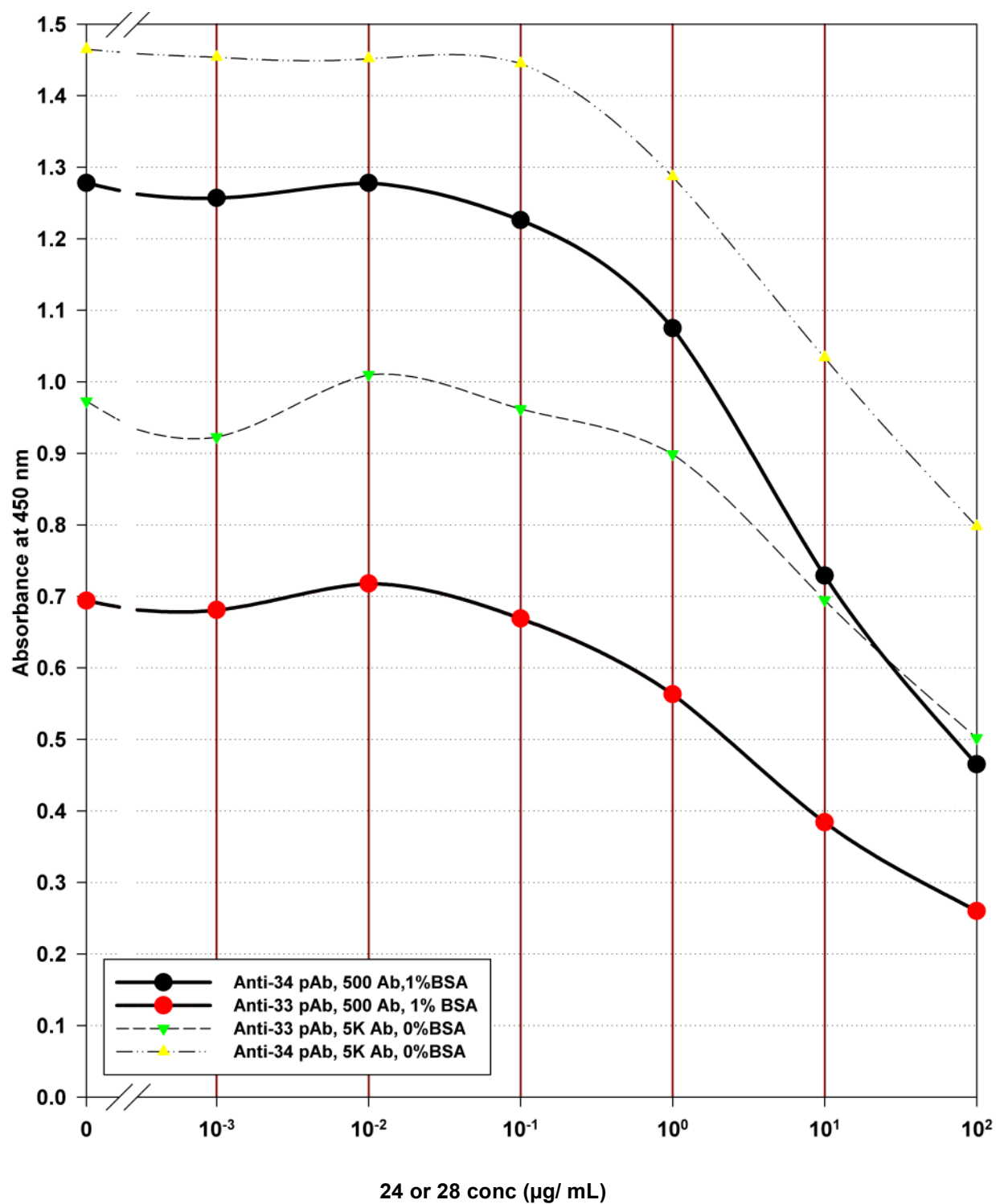


Figure 5.11 Standard curve of CI-ELISA's for **24** or **28** using anti-33 pAb or anti-34 pAb

HPLC-MS analysis showed a $[M+H]^+$ ion peak at m/z 1378.6751 corresponding to a molecular formula, $C_{70}H_{96}N_{11}O_{16}S$ of **29**.

1H NMR (500 MHz, $CDCl_3$, COSY, NOESY): Msn^1 : δ 4.76 (1H, m, α), 2.51 (1H, m, β), 2.39 (1H, m, β), 3.40 (2H, m, γ), 3.87 (2H, s, SO_2CH_2), 2.87 (4H, m, $NHCH_2CH_2NH$), 7.79 (1H, br s, $NHCH_2CH_2NHCoumarin$), 8.90 (1H, m, $NHCH_2CH_2NHCoumarin$), 3.91 (3H, s, OCH_3), 8.79 (1H, s), 7.57 (1 H, d, 8.6 Hz), 6.92 (1H, d, 8.6 Hz), 6.85 (1H, s), 7.31 (1H, m, NH); Leu 2 : δ 3.83 (1H, m, α), 1.93 (2H, m, β), 1.62 (1H, m, γ), 0.92 (6H, d, 7 Hz, δMe); Ile 3 : δ 4.47 (1H, m, α), 1.90 (1H, m, β), 1.49 (2H, m, γ), 1.07 (3H, d, 6.8 Hz, γMe), 0.82 (3H, t, 6.8 Hz, δMe); Pro 4 : δ 3.78 (1H, br s, α), 2.15 (1H, m, β), 1.64 (1H, m, β), 1.98 (1H, m, γ), 1.94 (1H, m, γ), 3.86 (1H, br s, δ), 3.61 (1H, m, δ); Pro 5 : δ 4.11 (1H, m, α), 1.93 (2H, m, β), 1.49 (1H, m, γ), 0.95 (1H, m, γ), 3.31 (2H, m, δ); Phe 6 : δ 4.86 (1H, m, α), 3.08 (1H, m, β), 2.98 (1H, m, β), 7.25 (m, 3H, δ , ϵ), 7.14 (2H, m, ζ); Phe 7 : δ 3.92 (1H, m, α), 3.31 (2H, m, β), 7.30 (m, 2H, δ), 7.25 (1H, m, ϵ), 7.14 (2H, m, ζ), 7.71 (1H, br s, NH); Val 8 : δ 3.86 (1H, m, α), 2.16 (1H, m, β), 0.90 (6H, d, 7 Hz, γMe); Ile 9 : δ 4.55 (1H, m, α), 1.98 (1H, m, β), 1.51 (2H, m, γ), 0.95 (3H, d, 6.4 Hz, γMe), 0.84 (3H, t, 6.4 Hz, δMe), 6.78 (1H, br s, NH).

^{13}C NMR (125 MHz, $CDCl_3$, HMQC, HMBC): Msn^1 : δ 52.1 (α), 25.5 (β), 50.6 (γ), 58.4 (SO_2CH_2), 40.5 ($NHCH_2CH_2NHCoumarin$), 39.5 ($NHCH_2CH_2NH_2$), 56.2 (OCH_3), 165.3, 163.2, 161.8, 161.2, 157.0, 148.6, 131.3, 114.9, 114.2, 112.6, 100.6, 171.0 (C=O); Leu 2 : δ 61.8 (α), 38.4 (β), 25.8 (γ), 23.1 (δMe), 21.9 (δMe), 171.7 (C=O); Ile 3 : δ 56.3 (α), 36.6 (β), 25.1 (γ), 15.6 (γMe), 11.1 (δMe), 171.9 (C=O); Pro 4 : δ 56.2 (α), 28.6 (β), 25.5 (γ), 48.1 (δ), 170.9 (C=O); Pro 5 : δ 61.1 (α), 32.0 (β), 21.7 (γ), 47.3 (δ), 173.7 (C=O); Phe 6 : δ 54.1 (α), 35.5 (β), 136.3 (γ), 129.7 (δ), 129.0 (ϵ), 127.3 (ζ), 173.2 (C=O); Phe 7 : δ 57.3 (α), 36.9 (β), 135.9 (γ), 129.1 (δ), 128.9 (ϵ), 127.3 (ζ), 175.4 (C=O); Val 8 : δ 56.2 (α), 29.4 (β), 19.7 (γMe), 18.3 (γMe), 173.2 (C=O); Ile 9 : δ 59.1 (α), 36.8 (β), 25.1 (γ), 16.1 (γMe), 11.3 (δMe), 173.0 (C=O).

Similarly, **27** (0.2 g, 0.18 mmol) was coupled with 7-methoxy coumarin-3-carboxylic succinimidyl ester (1.5 eq, 0.09 g) in presence of triethylamine (1eq, 0.19 mg, 0.025mL) in 30% methanol/acetonitrile solution to obtain **31** (0.25 g) using above procedure.

1H NMR (500 MHz, $CDCl_3$, COSY, NOESY): Msn^1 : δ 4.63 (1H, m, α), 2.52 (1H, m, β), 2.17 (1H, m, β), 3.29 (1H, m, γ), 3.13 (1H, m, γ), 3.72 (2H, s, SO_2CH_2), 3.60 (2H, m, $NHCH_2CH_2NHCoumarin$), 3.49 (2H, m, $NHCH_2CH_2NHCoumarin$), 8.76 (1H, br s,

NHCH₂CH₂NHCoumarin), 8.96 (1H, m, **NHCH₂CH₂NHCoumarin**), 3.87 (3H, s, OCH₃), 8.81 (1H, s), 7.63 (1 H, d, 9 Hz), 6.86 (1H, d, 9 Hz), 6.80 (1H, s), 7.74 (1H, m, NH); Leu²: δ 4.33 (1H, m, α), 2.33 (1H, m, β), 1.54 (1H, m, β), 1.84 (1H, m, γ), 0.83 (6H, m, δMe); Val³: δ 4.42 (1H, m, α), 2.17 (1H, m, β), 1.02 (6H, m, γMe), 7.56 (1H, br s, NH); Phe⁴: δ 4.42 (1H, m, α), 3.13 (1H, m, β), 3.06 (1H, m, β), 7.30-7.10 (m, 5H, δ, ε, ζ); Pro⁵: δ 4.12 (1H, m, α), 1.46 (2H, m, β), 1.59 (1H, m, γ), 1.46 (1H, m, γ), 3.29 (2H, m, δ); Leu⁶: δ 3.60 (1H, m, α), 1.70 (2H, m, β), 1.39 (1H, m, γ), 0.93 (6H, m, δMe), 7.95 (1H, d, 7.5, NH); Phe⁷: δ 4.97 (1H, m, α), 3.02 (2H, m, β), 7.30-7.10 (m, 5H, δ, ε, ζ); Ile⁸: δ 3.90 (1H, m, α), 1.76 (1H, m, β), 1.60 (2H, m, γ), 0.83 (3H, m, γMe), 0.93 (3H, m, δMe), 7.67 (1H, br s, NH).

¹³CNMR (125 MHz, CDCl₃, HMQC, HMBC): Msn¹: δ 52.0 (α), 25.4 (β), 50.7 (γ), 48.2 (SO₂CH₂), 40.3 (**NHCH₂CH₂NHCoumarin**), 38.7 (**NHCH₂CH₂NH₂**), 56.1 (OCH₃), 165.1, 162.9, 162.9, 161.9, 156.8, 148.8, 131.7, 114.7, 114.0, 112.8, 100.4, 172.8 (C=O); Leu²: δ 63.5 (α), 30.1 (β), 28.9 (γ), 22.9 (δMe), 21.7 (δMe), 172.8 (C=O); Val³: δ 57.7 (α), 30.9 (β), 19.5 (γMe), 18.4 (γMe), 171.9 (C=O); Phe⁴: δ 53.5 (α), 36.0 (β), 138.1 (γ), 129.1 (δ), 128.9 (ε), 127.0 (ζ), 173.2 (C=O); Pro⁵: δ 60.2 (α), 30.6 (β), 25.2 (γ), 46.4 (δ), 173.9 (C=O); Leu⁶: δ 61.2 (α), 40.2 (β), 25.0 (γ), 22.7 (δMe), 21.6 (δMe), 171.3 (C=O); Phe⁷: δ 53.5 (α), 37.6 (β), 137.7 (γ), 129.0 (δ), 128.4 (ε), 126.5 (ζ), 171.9 (C=O); Ile⁸: δ 58.7 (α), 35.9 (β), 25.2 (γ), 16.1 (γMe), 11.5 (δMe), 171.3 (C=O).

5.6 Conclusion

The analogs of CLs **10** and **11** containing amine and alcohol derivatives were successfully coupled to fluorescent dye, affinity matrix and protein. All the CL conjugates were characterized using different spectrometric techniques. CL dye conjugate was formed by covalent linkage of amino analogs with *N*-hydroxysuccinimide ester of coumarin dye. It shows that coumarin derivative of **11** exists as single isomer and **10** exist in two isomers. The fluorescence studies of CL dye conjugates were done in various solvents to determine the solvent effect. Spectroscopic analysis shows higher quantum yields for CL coumarin conjugates in methanol and ethanol as compare to acetonitrile and DMF. Amino analogs were coupled with *N*-hydroxysuccinimide ester of agarose gel to form CL affinity matrix. The coupling efficiency of affinity matrix to **11** analog was about 70% compare to **10** (38%). CL affinity matrix has shown binding to apolipoprotein A1 in chicken serum Apo A1 was characterized by the molecular mass

of 28 kDa obtained from the trypsin digest band. Hydroxyl analogs were coupled to BSA to elicit immune response and produce polyclonal antibodies in rabbits. The incorporation of CLs (haptens) was monitored by SDS-PAGE, MALDI and ESI-QTOF. Both MALDI and ESI-QTOF shows the attachment of haptens to BSA. In addition more than 25 haptens were incorporated to BSA in both CLs. Based on these results, the CI-ELISAs using the pAbs produced in this study could be applied to detect CLs in complex matrices. ELISA provides highly sensitive, rapid, and precise means to analyze a large number of samples. These purified antibodies can be potentially utilize in localizing CLs in cell lines and also can be used in detection of CLs in ageing flax. The use of tagged CLs will help to increase their utility before they can be effectively used in any application.

CHAPTER 6

GENERAL DISCUSSION

Plant cyclic peptides belong to the important class of biological active compounds. They possess various biological activities such as antimalarial, immunomodulating, immunosuppressive, antiplatelet and antifungal. However these peptides are found in very small quantity and the distribution varies between 10^{-2} to $10^{-5}\%$ of plant dry material (Tan & Zhou, 2006). This limits the utilization of peptides in various applications. The highest quantity of cyclic peptides is present in the seeds of *Annona glabra* ($1.0 \times 10^{-2}\%$), while *Phytolacca polyandra* ($1.4 \times 10^{-5}\%$) shows the lowest (Li, et al., 1999; Xiong, et al., 2002). Similarly CLs are also present in small quantity in flax for example, 30 kg of dry roots of flax have produced (**1** - 0.28 g; **2** - 0.016 g; **3** - 0.12 g; **4** - 0.048 g; **5** - 0.008 g) (Morita, et al., 1999). Another promising method to obtain peptides is by performing successive chemical reactions where single amino acids are added to the carboxylic end of a peptide or amino acid (Merrifield, 1963). However, the cost of synthesizing peptides using solid and solution phase chemistry is also high. For example, the cost of small-scale peptide synthesis (10 mg) ranges from roughly \$20–\$60 depending on the number of amino acids. As the number of amino acid residues increases, the cost also increases. Larger scale production of grams of peptide can lower the cost of production. A gram of a 40 amino acid peptide can cost just \$1600. (Latham, 1999). CLs could be utilized as drugs or drug delivery agents as they have biological activity, hydrophobicity and may form inclusion complexes. For example, the hydrophobicity of CLs plays a significant role in transport across cell membranes in addition to distribution in tissues and organs (Kemmer, et al., 1997) and helps in binding with human serum albumin (Rempel, et al., 2010). CLs also form inclusion complexes with metal ions such as Tb (Chatterji, et al., 1987) and forms crystalline solvates with alcohols (Jadhav, et al., 2011; Quail, et al., 2009; Schatte, et al., 2012). In this study we have taken advantage of the polarity of CLs by adsorbing on silica gel and solvent-solvent extraction

protocol was used to remove CLs from the silica column. However, tryptophan containing CLs such as CLs **4**, **6–9** were decomposed and HPLC-DAD showed presence of CLs **1**, **3**, **5**, **10** and **11**. We have oxidized the crude mixture to simplify the preparative chromatographic separation from five to three CLs **1**, **10** and **11** and obtain gram quantities. Previous process includes first obtaining pure CLs **3** and **5** and then oxidation to access **10** and **11**, which increases the number of purification steps (Matsumoto, et al., 2001).

The novel CLs **10** and **11** were completely characterized using NMR (1D and 2D) and LCMS. NMR experiments were done at different temperatures and well resolved sharp spectral signals were obtained from NMR acquisitions at 320 K. The results show that **11** exists as single isomer and **10** as two isomers. The temperature has effect on the NMR chemical shifts of CLs. In flax CLs, Pro residue plays a major role in the conformational flexibility and it can produce cis/trans isomers. These isomers convert at slower rate into each other to give separate signals that can complicate the spectra. Hence temperature variability is necessary to obtain well-resolved spectra without presence of minor conformers (Morita, et al., 1999). The variation in temperature can also be used to monitor the intramolecular hydrogen bonding of NH with the carbonyl oxygen. As the temperature increases the hydrogen bond is weakened and NH proton shifts to up field. It has been proposed that hydrogen bonded NH is found at downfield because of the interaction with carbonyl oxygen. However, this assumption is not always true. The proton exchange method can also be used to determine the hydrogen bonding (Baxter & Williamson, 1997).

These CLs **10** and **11** have shown thermal stability over 200 °C and there structures were confirmed by taking the NMR before and after the treatment. Normally, biological molecules such as proteins are only stable at physiological temp of around 40–45 °C (Vogt, et al., 1997) and there are also thermophiles proteins having stability above 100 °C (Scandurra, et al., 1998). But this thermal stability over 200 °C makes these CLs good candidates in the fields of biomedical, nanotubes technologies, microporous solids and photoswitches. For example, cyclic D, L peptides *cyclo*[-(Trp-D-Leu)₃-Gln-D-Leu-], shown to spontaneously self assemble in a columnar manner to be used in nanotubes and microporous solids. A small model cyclic phosphopeptides was tagged with hemithioindigo chromophore as photoswitch to study complex folding processes. (Afonso, et al., 2012; Cordes, et al., 2006; Ryu & Park, 2010). It was observed that **10** and **11** were not present in flaxseed oil and result of flax ageing. These pure compounds

can be used as standards in food industry for detection of ageing of flaxseed and flaxseed products.

The second aim was to utilize these gram quantities of CLs. Site directed chemical modification is often a formidable challenge and applicable to peptides that have few heteroatoms. (Kotha & Lahiri, 2005). This has advantage over linear peptide synthesis (Merrifield resin) as described above to perform SAR studies (Jamonnak, et al., 2010). We have described the chemical modification at Met side chain to synthesize active analogs, which are away from the bioactive tripeptide, Phe-Phe-Pro fragment (Kessler, et al., 1986). The chemical shifts of alpha proton of modified peptides relative to the parent gave important structural information about the conformation. (Dekan, et al., 2011). The analogs of **11** have no significant perturbations for most of amino acid residues in chemical shift, while analogs of **10** show little similarity. The reason might be hydrogen bonding of amino and hydroxyl analogs with the amide carbonyl. Met derivatives were oxidized to more stable Msn counterparts. Hence versatile active group at side chain can be used to attach different molecules and increase utility of CLs.

The final aim was to synthesis of CL adduct complexes such as fluorescent tagged CLs, affinity chromatography media, and haptens suitable for antibody production. The biological activity of CLs makes them good candidates to be used them as fluorescent tag. Fluorescent tags have been successfully used for imaging proteins inside cells and are valuable in establishing the fate of a given protein or chemical compound in biochemical processes (Ferro-Flores, et al., 2010). However there was no report on the attachment of fluorescent dye to CLs. In this study we are attaching a coumarin dye to CLs through a covalent linkage. NMR studies shows that CL dye conjugate molecules might have structural similarity with the parent molecule **10** and **11**. Hence they can be used for fluorescent imaging. We have developed a general synthetic protocol and could be used to prepare various fluorescent CL derivatives from commercial available dyes. The fluorescent CL compounds have shown similar structures as compare to parent CLs. This result shows that after coupling with the dye, CL conformation has not been altered and can be used for fluorescent imaging.

Protein-protein and protein-small solute interactions are important in many biological processes. Some of the examples include hormone binding with receptors, enzymes with substrates and binding of drugs and other compounds with serum proteins (Hage, 2002). Affinity chromatography plays important role in bioseparation of biomolecules from complex mixture. It

was reported that analogue of CL **1** have shown binding to proteins in the cytosol and plasma membranes of rat hepatocytes. The structural features important for binding with proteins were hydrophobic surfaces and cyclic conformational structure of CL (Kemmer, et al., 1997). We have made CL affinity matrix to study the binding of chicken serum proteins. In our study we have identified a specific binding of CL with protein which main function is to cholesterol efflux and reverse cholesterol transport. It was reported that CsA inhibits Apo A1 (Le, et al., 2004) and CLs binding with Apo A1 suggest that it may interfere in cholesterol homeostasis. Hence this study will help to understand the effect of binding on the biological activity of CLs.

Antibodies are generally produced from native or recombinant proteins. However peptides have also provided better platform to produce antibodies. They have added advantage such as manufacturing cost; easily produced with high purity and mimic the epitope of protein (Trier, et al., 2012). Peptide polyclonal antibodies are used as analytical tool as immunoblots to determine the native or denatured calreticulin in solution (Houen, et al., 1997). There are no reports on the generation of antibodies using CLs. Hydrophobic amino acids in CLs makes these CLs insoluble in water. But CLs were coupled with BSA in THF to form immunogen. The incorporation of haptens has successfully shown by MALDI and ESI-TOF. The generated pAbs in this study can be used in two ways: detection of CLs in flax and flaxseed products and therapeutic purpose for localization of CLs. Previous procedures for identification and characterization of these CLs were done using NMR, HPLC-MS methods (Matsumoto, et al., 2001; Morita, et al., 1999; Stefanowicz, 2001). However, they are not satisfactory for the detection of CLs in multi-component mixtures. In contrast, an enzyme-linked immunosorbent assay (ELISA) provides highly sensitive, rapid, and precise means to estimate biological parameters, with the added advantage that it can rapidly analyze a large number of samples.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Flax CLs are known to exhibit various biological activities such as immunosuppression and apoptotic induction in human epithelial cancer cell lines. CLs are thus bioactive compounds with potential for use as therapeutics. The current research investigated: 1) isolation of CLs from flax oil in multigram quantities, 2) the isolated CLs containing Mso were chemically modified by making specific changes to the Met or Mso group in a systematic fashion, without changing other amino acids, and 3) the modified CLs were linked to molecules or materials of interest including fluorescence tags, affinity chromatography media and BSA (for production of polyclonal antibodies). In addition, these CLs derivatives were completely characterized using spectrometric techniques including 1D and 2D NMR spectrometry, as well as mass spectrometry.

Methods have been developed to isolate and purify five CLs namely **1**, **3**, **5**, **10** and **11** from flax oil in multigram quantities. We have developed an oxidation protocol to simplify the purification step by reducing the number of CLs from five to three (**1**, **10** and **11**). CLs **10** and **11** have been fully characterized using NMR methods and their thermal stability has been reported. In addition the solution state structure of **11** is similar to its solid state and shows presence of single conformer. These solid and solution state studies will lead us to determine the binding region of the CLs. However, crystals could not be grown for **10** and NMR also shows presence of two isomers. Prior to this study, there were not sufficient CLs available to perform chemical modification and structure activity relationship studies. These CLs have to be synthesized using solid phase resin method that leads to increase in the overall cost of the material.

A synthetic strategy has been developed to chemically modify the Met side chains of the parent CLs. These Met CLs can be obtained by reduction of Mso group. The modification through Met is not known and mostly reactive amino acids such as lysine, aspartic acid, glutamic acid serine and threonine have been used as point of attachment to other functional group. The

synthetic reactive analogs containing active amine and hydroxyl have been synthesized and completely characterized using 1D and 2D NMR techniques. The thermal stability of Msn CLs has been utilized by oxidizing the modified Met.

These reactive analogs have been successfully coupled with fluorescent moieties for use as fluorescent tags. A coumarin dye was added to the CL side chain and their spectroscopic analyses have shown high quantum yields. In addition, the CLs have been fused to affinity chromatography media to purify CL-binding proteins from complex mixtures. For example, they have shown affinity to the apolipoprotein A1 present in the chicken serum and finally, CLs were also coupled with protein to act as haptens to produce antibodies. These antibodies were used to develop competitive indirect ELISA.

Key findings and highlights of this research include the following:

- The present research relates to methods for isolating CLs from flaxseed by simplifying the CL extracts to increase the efficiency of chromatography for recovery of specific CL(s).
- CLs **10** and **11** were found to be ageing products of flax oil and solution structures were determined. Hence they can act as standards in the food industry as markers of oxidation.
- The CLs **10** and **11** have been shown to have suitable heat and oxidative stability for use in harsh environments. This makes them good candidates in the fields of nanotubes, biomedical and other microporous materials.
- A new branch of CL modification through sulfur chemistry has been developed.
- Methionine derivatives were stabilized by oxidation to the sulfone. It was found that modified Met and Mso side chain were easily eliminated to alkane and vinyl compounds.
- CLs are readily linked to fluorophores, proteins and solid matrices through modifications to the methionine.
- CL-conjugates were completely characterized using NMR and MS methods. These CL conjugates were successfully tested in various applications.

This work has provided a general protocol to isolate, extract and chemically modify CLs. The availability of sufficient quantities will also allow for chemical modification of naturally isolated CLs and carrying out biological assays to establish potential pharmaceutical applications. The modification of the CLs will be necessary in order to increase their utility

before they can be effectively used in any application. The above examples clearly show that these CLs can be used in the fields of fluorescence, affinity purification and producing antibodies. This methodology can also be used to generate various analogs for structure activity relationship studies. These applications will be a value added products for the flax industry because it will benefit both farmers and consumers. The extraction protocol can be used to remove the bitter flavour CL **5** from flax oil and these CLs can be sold as nutraceuticals.

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